Notice of Opposition to a European Patent

To the European Patent Office

Tabulation marks

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EPO - Munich 51
14. Juni 2005

I. Patent opposed

Opp. No. OPPO (1)

Patent No.	0 656 786 B1
Application No.	93909679.8
Date of mention of the grant in the European Patent Bulletin (Art. 97(4), 99(1) EPC)	15.09.2004

Title of the invention:

USE OF ISOFLAVONE PHYTO-OESTROGEN EXTRACTS OF SOY OR CLOVER

II. Proprietor of the Patent

KELLY, Graham Edmund

first named in the patent specification

Opponent's or representative's reference (max. 15 spaces) O1427EP OREF

III. Opponent

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Multiple opponents: [X] further opponents see additional sheet

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2. Employee(s) of the opponent authorised for these opposition proceedings under Art. 133(3) EPC

Name(s):

Authorisation(s)

To 1./2.

[X] not considered necessary
[ ] has/have been registered under No.
[ ] is/are enclosed

EPO Form 2300.1 04.93 (int. ed. 12/97)

V. Opposition is filed against

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— the patent as a whole [X]

— claim(s) No(s).

VI. Grounds for opposition:

Opposition is based on the following grounds:

(a) the subject-matter of the European patent opposed is not patentable (Art. 100(a) EPC) because:

— it is not new (Art. 52(1); 54 EPC) [X]

— it does not involve an inventive step (Art. 52(1); 56 EPC) [X]

— patentability is excluded on other grounds, i. e. Art. [ ]

(b) the patent opposed does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art (Art. 100(b) EPC; see Art. 83 EPC). [X]

(c) the subject-matter of the patent opposed extends beyond the content of the application/ of the earlier application as filed (Art. 100(c) EPC, see Art. 123(2) EPC). [X]

VII. Facts and arguments

(Rule 55(c) EPC)
presented in support of the opposition are submitted herewith on a separate sheet (annex 1) [X]

VIII. Other requests:

Oral proceedings Art. 116(1) EPC

IX. Evidence presented

for EPO use only

Enclosed = X

will be filed at a later date =

A. **Publications:**

Publication date

1 SEE LIST OF ENCLOSURES

Particular relevance (page, column, line, fig.):

2

Particular relevance (page, column, line, fig.):

3

Particular relevance (page, column, line, fig.):

4

Particular relevance (page, column, line, fig.):

5

Particular relevance (page, column, line, fig.):

6

Particular relevance (page, column, line, fig.):

7

Particular relevance (page, column, line, fig.):

Continued on additional sheet

B. **Other evidence**

Continued on additional sheet

X. Payment of the opposition fee is made

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[X] as indicated in the enclosed voucher for payment of fees and costs (EPO Form 1010)

[ ]

XI. List of documents

Enclosure No.		No. of copies	
0	[X] Form for notice of opposition	2 (min. 2)	
1	[X] Facts and arguments (see VII.)	2 (min. 2)	
2	Copies of documents presented as evidence (see IX.)		
2a	[X] – Publications	2 (min. 2 of each)	
2b	[ ] – Other documents	(min. 2 of each)	
3	[ ] Signed authorisation(s) (see IV.)		
4	[X] Voucher for payment of fees and costs (see X.)	1	
5	[ ] Cheque		
6	[X] Additional sheet(s)	2 (min. 2 of each)	
7	[X] Other (please specify here): Form 1037 Aknowledgement of Receipt		

XII. Signature of opponent or representative

Place Madrid

Date 13 June 2005

Please type name under signature. In the case of legal persons, the position which the person signing holds within the company should also be typed.

Additional Sheet to Notice of Opposition

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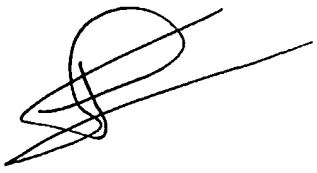

OPPOSITION TO EP 0 656 786 B1

ANNEX 1

STATEMENT OF FACTS AND ARGUMENTS

1. ART. 100(a) EPC: THE SUBJECT-MATTER OF EP 0 656 786 B1 IS NOT PATENTABLE WITHIN THE TERMS OF ART. 52 TO 57 EPC

1.1 The priority is not valid for the claims of EP 0 656 786 B1

A fair reading of the priority application PL2511 (**D3**), filed on 19.05.1992, shows that the invention as claimed in EP 656 786 B1 is not entitled to the priority right.

The reasons are as follow:

- There is no reference in the Australian provisional application to an "isoflavone phyto-oestrogen extract of soy". When soy is mentioned as a source for the phyto-oestrogens, it is always limited to soya hulls, soya hypocotyls or a mixture of both (see page 7, lines 6-12; page 8, lines 14-24; page 9 line 3; page 10, lines 26-35, page 12, lines 3-5, examples 1 and 3). There is no basis for the generalisation to "extract of soy". Although line 8 at page 9 reads "*while soya is the preferred source of phyto-oestrogens...*", it has to be read not in isolation but in the context of the whole paragraph. It says that the reason to prefer soya is because the ease in separation and collection of hulls and hypocotyls. The priority document does not show directly and unambiguously that any other soy source than hulls and hypocotyls was contemplated.

- There is no disclosure either of a "isoflavone phyto-oestrogen extract of clover". Only one species of clover is disclosed as alternative source for the phyto-oestrogen compounds, i.e. Subterranean clover (see page 9, lines 16-21). The term clover is more general, it is a genus that includes all clover

species, "extract of clover" is a generalisation that was not present in the priority document.

- When the application discloses methods of treatment of conditions associated with phyto-oestrogen deficiency, it says *"administration of an effective amount of phyto-oestrogen selected from the isoflavones and/or coumestan, ideally in concentrated form"* (see page 11, line 32- page 12, line 2). It includes an amount limitation ("effective amount") which is not present in claim 1 of EP 656 786, in any case it does not mention the "isoflavone phyto-oestrogen extract of soy or clover" for the method of treatment.

- The "administration in unit dosage form" present in claim 1 is not present in the priority document, only the particular embodiments tablet, capsule and "*convenient dosage forms*" are mentioned (page 12, lines 19-29).

Therefore several essential features of claim 1 are not present in the priority document. According to **G 2/98** a strict interpretation of what is meant by "the same invention" in art. 87(1) EPC (priority right) must be followed:

"The requirement for claiming priority of "the same invention", referred to in Article 87(1) EPC, means that priority of a previous application in respect of a claim in a European patent application in accordance with Article 88 EPC is to be acknowledged only if the skilled person can derive the subject-matter of the claim directly and unambiguously, using common general knowledge, from the previous application as a whole."

The common general knowledge referred to in **G2/98** cannot be used to add features, generalise, or complement what has been disclosed in the priority document, but only to determine what is directly and unambiguously derivable from this document.

It is clear from the above that the subject-matter of claim 1 of EP 656 786 B1 cannot be derived directly and unambiguously from the priority document, it is not the same invention in the sense of art 87(1) EPC. This also applies for all

dependent claims (2-11) because they refer back to claim 1. In addition, some of the features in these claims were not present in the priority document either.

Therefore, for the purpose of art. 54 EPC, the provisions of Art. 89 EPC do not apply and the date of filing for this patent should be **19.05.1993** and not 19.05.1992.

NOVELTY

1.2 Lack of Novelty pursuant to Art 54(3) and (4) EPC

The application **WO 94/23716** (**D4**) was filed on 15.04.1994 claiming priority from US 08/049,006 (**D5**) filed 16.04.1993 (i.e. before the filing date of EP 656 786 B1). The content of the PCT application and its priority document is nearly the same, the priority is valid. This application entered the European regional phase on 11.10.1995 designating and paying the fees for 16 contracting states, the same as those of EP 656 786 B1 with the exception of Monaco. Therefore, according to Art. 54(3) and (4) EPC, the contents of WO 94/27313 (**D4**) shall be considered as comprised in the state of the art for the assessment of novelty.

Comparing the content of the claims of EP 0656 786 B1 with this document:

Claim 1:

D4 discloses the use of an isoflavonoid in the preparation of a medicament for preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen (see claim 1). According to page 1, lines 5-7, the treatment are therapies for the prevention and treatment of menopausal and premenstrual syndroms. D4 states that "*The isoflavonoid may be administered in the form of a plant extract rich in isoflavonoids*" (page 2, lines 9-10), "*accordingly, an isoflavonoid-containing fraction can be extracted from a soy or plant product, It is preferred that the isoflavonoids be extracted and concentrated from soy bean or soy powder*" (page 3, lines 1-4). The

medicament form is also disclosed at page 3, line 31. The unit dosage form is disclosed in claim 3.

Therefore D4 takes away the novelty of claim 1, because all the features of this claim are present in combination in this application.

Claim 2:

D4 also discloses the dietary suitable excipient of claim 2, see page 3, lines 6-11 (palatable carrier, dietary product).

Claim 3:

D4 discloses soya as the source of the isoflavonoid fraction, the features of claim 3 are also anticipated.

Claim 6:

The particular isoflavones genistein or daidzein are also mentioned in D4, see page 1, line 35 and page 3, lines 16-19.

Claim 7:

Claim 5 of D4 discloses a ratio of genistein:daidzein of 10-30 mg : 10-5 mg, i.e. 1:1 to 6:1, thus claim 7 is also anticipated because some of the values fall into the claimed range.

Claim 8:

D4 discloses the dose of at least 30 mg isoflavonoids (see claim 3), i.e. within the rage of 20-200 mg of claim 8 of EP 656 786 B1.

Claim 11:

D4 also states that the medicament can be in the form of a tablet (page 3, lines 31-32).

In conclusion, the subject-matter of claims 1-3, 6-8 and 11 lack novelty according to the provisions of art. 54 EPC because it is disclosed by D4.

1.3 Lack of Novelty pursuant ot Art. 54 (1) and (2) EPC

Document **D6** (Beckham, N. *Australian Wellbeing*, no. 29, 1988, pages 74-76: "Herbal Help to avoid Menopause Symptoms") discloses that phyto-oestrogens present in herbal remedies have an hormonal effect (page 74, middle column, last paragraph) usxeful to treat menopausal symptoms. Among the herbal remedies disclosed are red clover, which is said to be sold as an herbal tea. An herbal tea is made of leaves or parts of the plants in dried form, therefore it is an "extract" in the sense of claim 1 of EP 656 786 B1. In example 2 of the opposed patent an "extract" of soy is made by drying and separating whole soy hypocotyls without any further chemical separation step.

The phyto-oestrogens present in the red clover herbal tea mentioned in D6 are not characterized as "isoflavones", but since they are said to have oestrogenic effect, and the tea is from red clover it is implicitly disclosed that the oestrogens referred to are isoflavones. Moereover, at page 75, last 3 lines, genistein is explicitly mentioned as a "weak plant oestrogen".

D6 does not use the term "medicament" or "unit dosage form". These terms, present in claim 1 of EP 656 786 B1, have to be interpreted in the light of the description according to art. 69 EPC. In particular at page 7, lines 23-28:

"*The medicaments of the invention may be presented as..drinks,..lotions, pastes, gels or the like.*"

and in particular line 28:

"The medicaments are preferably presented as herbal remedies and treatments".

Therefore herbal remedies, such as those disclosed by D6 fall under the term "medicament" encompassed by claim 1. It follows that D6 discloses all the features of present claims 1 and 5, consequently these claims are not novel (Art. 54 EPC).

INVENTIVE STEP

1.4 The subject matter of the claims is obvious in view of the state of the art at the filing date of EP 656 786 B1.

Using the problem-solution approach to assess inventive step (Guidelines, C-IV, 9.8):

Document **D6** is considered the closest state of the art for the soy part of claim 1 because it has the same purpose and effect as the subject-matter of claim 1 and relates to the same problem as the one discussed in the introduction of the opposed patent (see EP 656 786 B1, page 4, line 55 to page 5, line 13).

D6 discloses the use of herbal remedies containing phyto-oestrogens that induce an hormonal effect in humans in order to treat symptoms associated with the menopause (page 74, middle column). Besides red clover, which was discussed above under novelty, it also proposes to use soya beans and states:

"*Sprouts are the best way to have these, particularly as the sprouting dramatically increases the oestrogen content*" (page 75, middle column, lines 7-11."

Thus D6 proposes soy products for the treatment of menopause symptoms due to their oestrogen content, and points to the need of having a sufficient content of oestrogens. As mentioned before, the term plant oestrogen is used in D6 instead of isoflavone phyto-oestrogen. This is clear from the mentioning of red clover or soya as sources, and also of genistein as having activity in oestradiol receptors at the end of page 75.

D6 already states the problem of using soya beans and soy sprouts as a source of isoflavones to achieve an oestrogenic effect: soya beans are not appetising, in particular if people don't normally eat them, and soya sprouts, that have the advantage of a higher content in oestrogens, are difficult to sprout (page 75, middle column, lines 1-34).

Since herbal remedies fall under the definition of medicament according to EP 656 786 B1, the only difference of claim 1 with the disclosure of D6 is that an "extract" of soy is used instead of soy beans or soy sprouts. The term "extract" is not defined in EP 656 786 B1. However, it appears from the patent specification to be a large concept that comprises, among others, physical separation or chemical extraction.

In view of D6, the objective technical problem faced by the person skilled in the art is to provide an alternative source of soy oestrogens sufficient to produce an hormonal effect, rather than the soy food sources already proposed in D6.

The solution proposed by claim 1 to this problem is to use an "isoflavone phyto-oestrogen extract of soy".

D6 already points to such an alternative: isolated extracts of alfalfa in the form of tablets are disclosed as alternatives to alfalfa sprouts, see page 74, lines 29-30. The author of D6, being a natural therapist, does not recommend plant extracts because she sees them as "drugs" and recommends food sources instead. But the alternative is there and was well known at the time. Thus, in view of D6 it was obvious for the person skilled in the art that a soy "extract" containing sufficient isoflavone could be used instead of the soy sprouts.

During examination the patentee alleged that at the filing date there was a technical prejudice against the use of isoflavones phyto-oestrogen extracts of soy or clover. He bases this argument in the document **D7** (Kaldas and Hughes *Reproductive Toxicology Review* ,1989, 3(2), 81-89) stating that it discloses the deleterious effects of isoflavones on humans and animals (see letter of 21.07.2003, page 3).

According to **T341/94**, reasons 6.1.1:

"a prejudice in any particular field relates to an opinion or preconceived idea widely or universally held by experts in that particular field. ... The prejudice must have existed at the priority date; ..."

Document D7 discusses mainly animal studies and problems associated to phyto-oestrogen consumption by herbivores, such as the clover-diseased ewes, feed exclusively on pasture, or to the acute administration to rodents of high doses of some phyto-oestrogens. There is little discussion of evidence in humans, such as at page, 85, middle of left-hand column, when the authors state; *"it is plausible that human vegetarians may have ovulatory dysfunction but suffer no other obvious physiologic abnormalities due to their diets"*.

At page 88, when discussing human diseases, the possible deleterious roles of phyto-oestrogens are always proposed as speculative suggestions: *"have been suggested"*, *"may be correlated"*, *"could be a factor"*.

But when talking about the beneficial roles they are stated positively: *"A final beneficial phytooestrogenic effect is alleviation of vasomotor symptoms in menopausal women. Historically the Chinese have used herbal medicine to treat "hot flushes". These herbal medications work as well as Premarin (an equine conjugated oestrogen) in the mitigation of these symptoms in women with natural menopause (38)"*.

So when the author concludes that the majority of the effects of phytoestrogens are nocuous for mammals, either he refers to the effects on grazing animals or, if he refers at all to humans, he is just expressing his personal view. Document D7 does not demonstrate that it is an idea widely or universally held by the experts at the time of filing.

As stated in the Case Law of the Boards of Appeal of the EPO 4th Ed. 2001, page 134:

"prejudice cannot be demonstrated by a statement in a single patent specification, since the technical information in a patent specification or a

scientific article might be based on special premises or on the personal view of the author".

As a matter of fact, D7 actually reinforces the idea that phytooestrogens were used for a long time as herbal remedies for the treatment of menopausal symptoms such as "hot flushes".

More representative of the state of the art concerning safety issues at the time of filing are the following documents:

Document **D8** (Wilcox et al. *BMJ*, vol. 301, 1990, pages 905-906 : "Oestrogenic effect of plant foods in postmenopausal women") reports a study with 25 postmenopausal women not taking oestrogen replacement therapy and fed with a diet supplemented with soya flour, red clover sprouts and linseed (top of page 906). No adverse event is reported, but an oestrogenic effect was seen.

This study is most probably the same as the one referred to as forming part of the state of the art by the opposed patent at page 4, lines 48-51:

"*In one example, the diets of women, with menopausal syndrome were supplemented with foodstuffs (soya, linseed, red clover) high in phyto-oestrogens, and an alleviation of menopausal symptoms to an extent similar to that obtained with replacement therapy with synthetic oestrogens was achieved; that effect was ascribed to the phyto-oestrogen content of the supplement*."

Document **D9** (Messina M., Barnes S., *J NCI*, 1991, pages 541-546 : "The role of Soy Products in Reducing Risk of Cancer") reports at page 542 (paragraph bridging left and right column) a study by Baird on postmenopausal women that were given soy food products daily over a period of 4 weeks, with an estimated isoflavone content of 200 mg/day, in order to study the estrogenic effect. The women that were fed soy in these doses exhibited an estrogenic response.

Significantly, no negative effect is reported, even at such daily doses for a period of 4 weeks.

Document **D10** (Adlercreutz et al. *Am J Clin Nutr*, 1991 54:1093-1100, "Urinary excretion of lignans and isoflavonoid phyto-oestrogens in Japanese men and women consuming a traditional Japanese diet") describes study in Japanese people consuming high amounts of soybean products. The very high excretion of isoflavonoids correlated with soybean intake. At page 1097 several possible advantages are discussed, such as a role in the prevention of prostate cancer. Even a study demonstrating the prostate cancer prevention is reported: "*Santti's group in Turku, Finland, in a collaborative study with us, observed that dietary soy prevented the development of precancerous changes in a neonatally estrogenized mouse used as a model for prostatic cancer (69)*". Adlercreutz was a well known researcher in the field. Again, there is no warning against any harmful effects of isoflavonoids.

Document **D11** (EP 135 172 A) discloses medicaments containing isoflavone compounds such as Daidzein (compound I) or Genistein (compound II) for the treatmen of osteoporosis as a result of hypoovarianism (the same cause of menopausal symptoms). The toxicity test example 5 at pages 7 and 8 shows that even at very high doses toxicity was not seen to the point that the maximum tolerated dose could not be calculated.

Therefore, at the time of filing the person skilled in the art was not prevented against the use of "extracts" of soy because they were not considered toxic, neither high doses of soy food or the pure compounds had any adverse effect that would hold him from applying the obvious solution. There was no technical prejudice against using isoflavones phyto-oestrogen extracts in humans, for example at daily doses such as 200 mg.

As **T341/94** concludes in a similar situation at reasons 6.1.1:

"A prejudice in the field of oral compositions must not be confused with a reasonable fear regarding the safety of a product which has not yet been clinically tested. Such a fear can be dispelled with appropriate clinical tests, a view expressed by Newbrunn in his declaration dated 10 September 1993."

From all the above it can be concluded that the person skilled in the art, starting from D6 and willing to avoid the problems that food sources of soy isoflavones generate, will have find it obvious to use "extracts" instead, in view that such alternatives are already proposed for other plants such as red clover (herbal tea, i.e. dried leaves) or alfalfa (extracts, tablets). The state of the art at the time of filing would not have prevented him from trying this alternative, in view that different sources of soy isoflavones (pure, as food, or food products) in different doses, including high doses, were safe and had been given to humans without reporting any adverse effect. The state of the art would have encouraged him to do so, in view of the many references to the beneficial effects of the use of soy isoflavones in the treatment of menopause symptoms or prostate cancer.

The claimed invention adds nothing beyond the teaching of the state of the art at the time of filing. Therefore claim 1 of EP 656 786 lacks inventive step (art. 52 and 56 EPC).

Claim 2

The use of a dietary suitable excipient in addtion to the extract cannot serve as basis for the presence of inventive step. If the "medicament" is to be given as a food supplement, there is nothing exceptional in using such excipients. For example in the study of Baird referred at page 542 of D9, the soy product is given as a spread for snackers, thus carrying dietary suitable excipients.

Claims 3 and 4

The use of soya or soya hypocotyls is suggested by D9 as well, see page 544, last paragraph of left-hand column. It states the well known fact that soy hypocotyls concentrate the majority of isoflavones, it is obvious that this will be an advantageous source of soy isoflavones requiring less extraction effort.

Claim 5

Besides herbal tea as mentioned under novelty, any other form of "extract" of red clover will be an obvious alternative: D6 discloses use of red clover for the treatment of menopause symptoms, other forms extracts (tablets, etc) will be seen as obvious alternatives as in the case of soya.

Claim 6

The particular isoflavones listed will be implicit in any isoflavone phyto-oestrogen soy extract. See for example **D12** (Eldridge et al. *J Agric Food Chem.* Pages 394-396, "Soybean isoflavones: Effect of Environment and Variety on composition").

Claim 7

The range for the ratio of particular isoflavones has no particular advantage, it reflects the proportions of the phyto-oestrogens isoflavones present in sources such as soy or red clover. See for example **D12**, at page 395, table II. It shows the amounts of genistein and daidzein and also that they are more concentrated in the hypocotyls. Thus this claim it will also be obvious for the skilled person.

Claim 8

D9 shows that an isoflavone amount of 200 mg/day causes an oestrogenic effect, rendering obvious the range of dependent claim 8.

Claim 9

D6 already mentions the daily treatment to treat menopause symptoms (see page 75, middle column, 6th paragraph. D8 and D9 also discloses the daily treatment for 6 and 4 weeks respectively. Therefore the subject matter of claim 9 is also obvious in the light of the prior art.

Claim 10

D6 mentions the use of soya sprouts that are known to contain coumestans. Document D9 suggests the use of soy products rather that isolated compounds (page 545) because the different components can also be responsible of a beneficial effect. Thus the subject matter of present claim is also obvious in view of the state of the art. Moreover, the opposed patent does not describe any particular problem solved by the combined use of coumestans, lignans and flavones.

Claim 11

The presentation of a plant extract in the form of a tablet is already disclosed in D6. Further, tablets or capsules are convenient dosage forms as alternatives to food, see also D11, end of page 7.

As a conclusion, it is submitted that all the claims of the opposed patent do not meet the requirements of art. 56 EPC because their subject-matter is rendered obvious by the prior art at the time of filing.

1.5 Lack of Inventive Step because the claimed invention does not work over the whole area claimed (T939/92)

The examining division recognised inventive step on the basis of the additional experimental data (GK1, GK2) submitted by the patentee on October 21 2003, according the the annex to the communication under R51(4) EPC.

GK1 was filed on 21.10.2003 together with a statutory declaration by the inventor of the opposed patent, G.E.Kelly (see **D13**). Item 3 of the statutory declaration refers to the examiner's report of 11.08.2003, thus the declaration was done after this date. Item 4 states:

" *4. I attach marked Exhibit **GK1** a copy of a declaration I made in connection with prosecution of a corresponding United States application. As set out in that*

declaration, the compositions/medicaments referred to in the claims of this European application have been shown in patient studies to be effective in the treatment of prostate cancer, premenstrual syndrome and menopause. These were patient studies in human subjects afflicted with the disorders of prostate cancer, premenstrual syndrome and menopause."

GK1, dated 3.3.1997, describes those studies. At page 3 it describes the treatment of 8 menopausal women treated in two groups with 40 mg or 160 mg of the inventive composition administered orally on a daily basis. 4 more women were treated with placebo. The inventive composition is said at the top of page two to be in accordance to examples 1 or 2 of the patent, i.e. red clover product or soya hypocotyl product. Hot flushes, night sweats, Greene Score, vaginal pH, vaginal cytology and mean cholesterol levels were measured as indicators. It states:

"*A significant change in menstrual symptoms was observed and a dose response change was observed between the 40 mg and 160 mg dosage range. This indicating that 160 mg per day was the most effective dosage for treatment of menopausal symptoms*".

Document **D14** (Knight et al, *Climacteric*, 1999, 2:79-84 "The effect of Promensil, an isoflavone extract, on menopausal symptoms") reports a randomized, double blind, placebo controlled trial of postmenopausal women carried out in Australia and sponsored by Novogen. They were treated with placebo or with tablets of 40 mg or 160 mg isoflavone containing red clover extract (Promensil, Novogen). We haven't found any other reference of a published clinical trial with these amounts of red clover extract. This clinical trial measures the same indicators (greene score, etc) as mentioned previously. The published conclusion of the trial is that there was no significant difference between the placebo group and the group on isoflavones, and that there was a a large placebo response. In any case it does not show that a composition in

accordance with the invention is effective in the treament of symptoms associated with menopause.

It is significant that the result of this trial was available in 1999, well before the statutory declaration of G.E. Kelly in 2003.

Document **D15** (Van de Weijer, *Maturitas* 42 (2002) 187-193 : Isoflavones from red clover (Promensil) significantly reduce menopausal hot flush symptoms compared with placebo") was cited by the patentee and reports a placebo controlled trial with 15 women on 80/mg isoflavone extract from red clover and 11 on placebo. Positive results are reported. But at page 191, left hand column, last paragraph, the author suggests that the dose might play a role in the results in comparison with other trials showing negative results.

Document **D16** (Tice et al., *JAMA* July 2003, pages 207-213 : "Phytoestrogen Supplements for the treatment of Hot Flashes : the Isoflavone Clover Extract (ICE) Study") reports a clinical trial funded by Novogen with 246 women recently postmenopausal. The amounts of isoflavone given were aprox. 82 mg and 57 mg. It was designed with a placebo run-in phase to take into account the placebo effect. It concludes that "*although the study provides some evidence for a biological effect of Promensil, neither supplement had a clinically important effect on hot flashes or other symptoms of menopause*". At page 212, center and right column the author speculates that the dose or the composition might be relevant to obtain a clinically important effect.

From the above referred clinical trials (in particular the larger one) that have been sponsored by the patentee, it is clear that the efficacy of a medicament for the treatment of symptoms associated with menopause and comprising an "isoflavone phyto-oestrogen extract of red clover" has not been proven. It ensues, following **T939/92** that the technical problem that the opposed patent

addresses is not solved over the whole breath of the claim and the requirements of art. 56 EPC are not met. The headnote of **T939/92** reads:

"1. If a claim concerns a group of chemical compounds per se, an objection of lack of support by the description pursuant to Article 84 EPC cannot properly be raised for the sole reason that the description does not contain sufficient information in order to make it credible that an alleged technical effect (which is not, however, a part of the definition of the claimed compounds) is obtained by all the compounds claimed (see reasons 2.2.2).

2. The question as to whether or not such a technical effect is achieved by all the chemical compounds covered by such a claim may properly arise under Article 56 EPC, if this technical effect turns out to be the sole reason for the alleged inventiveness of these compounds (reasons 2.4 to 2.6)."

The following point from the same decision explains clearly the reason for this:

"2.4.2. The reason for this is, that it has for long been a generally accepted legal principle that the extent of the patent monopoly should correspond to and be justified by the ***technical contribution*** *to the art (see T 409/91, OJ EPO , No. 3.3. and 3.4 of the reasons, and T 435/91, OJ EPO 1995, 188, reasons No. 2.2.1 and 2.2.2). Now, whereas in both the above decisions this general legal principle was applied in relation to the extent of the patent protection that was justified by reference to the requirements of Articles 83 and 84 EPC, the same legal principle also governs the decision that is required to be made under Article 56 EPC, for everything falling within a valid claim has to be inventive. If this is not the case, the claim must be amended so as to exclude obvious subject-matter in order to justify the monopoly."*

2. ART 100 (b) EPC: EP 0 656 786 B1 DOES NOT DISCLOSE THE INVENTION IN A MANNER SUFFICIENTLY CLEAR AND COMPLETE FOR IT TO BE CARRIED OUT BY A PERSON SKILLED IN THE ART

From the discussion under inventive step it appears that the invention does not work over the whole breath of the claim. According to the headnote of **T435/91**:

"*The disclosure of an invention relating to a composition of matter, a component of which is defined by its function (in the present case an additive which forces a detergent composition in the hexagonal liquid crystal phase), is not sufficient if the patent discloses only isolated examples, but fails to disclose, taking into account, if necessary, the relevant common general knowledge, any technical concept fit for generalisation, which would enable the skilled person to achieve the envisaged result without undue difficulty within the whole ambit of the claim containing the "functional" definition (point 2.2.1 of the Reasons)*".

In the present case claim 1 is very broadly formulated: the use of any isoflavone containing "extract" of soy or clover (any clover) to manufacture a "medicament" for the treatment of the 3 mentioned indications is covered. As already explained the terms "extract" and "medicament" are extremely large as well, from their interpretation in the description of the opposed patent. The patentee has failed to prove that the therapeutic effect can be achieved for any amount of isoflavone and for any kind of soy or clover extract. In view of the diverse range of amounts of isoflavones present in different plants species and in the different "extracts" possible, and in view of the prior art and publications that question the effectivity of some of these extracts, it is submitted that the invention as defined by the claims is not sufficiently disclosed for it to be carried out by a person skilled in the art (art. 83 EPC).

3. ART 100 (c) EPC: THE SUBJECT-MATTER OF EP 0 656 786 B1 EXTENDS BEYOND THE CONTENT OF THE APPLICATION AS FILED

It is submitted that subject-matter which extends beyond the content of the application as filed (WO 93/23069) has been introduced by way of amendments into claim 1 and the description of EP 0656 786 B1, contrary to the provisions of Art. 123(2) EPC. The reasons are as follows:

3.1 There is no basis for the feature "*treatment of prostate cancer*" of claim 1.

Claim 1 defines: "*The use of an isoflavone phyto-oestrogen extract of soy or clover for the manufacture of a medicament for administration in unit dosage form for the treatment of pre-menstrual syndrome, symptoms associated with menopause, or prostate cancer.*"

In the application as filed prostate cancer is mentioned in two contexts:

- At page 8, lines 17-21, "*amelioration of prostate cancer*" is specifically mentioned, "*by administering to the human a sufficient amount of phyto-oestrogen*". According to the applicant the administration of a sufficient amount of phyto-oestrogen is essential to ameliorate prostate cancer. At page 14 line 12 this is stated again: "*If desired greater dosages can be administered for therapeutic reasons*". In present claim 1 the limitation to "*sufficient amount*" that should be linked to the prostate cancer treatment, is not present, resulting in an undue generalisation.

- At page 15, last line the reduction of the risk of development of cancer of the prostate is disclosed in the following context:

"*The product of the invention modulates the production and/or function of endogenous sex hormones in humans to modify or producing health improving*

effects, including the following: ...(ii) reduced risk of development of cancer of the prostate; ...".

Further, at page 16, third paragraph, when prostate cancer is discussed, only the protection from development of prostatic cancer is mentioned. There is a big difference between avoiding (preventing) the development of prostate cancer and treatment of a prostate cancer which is already present. Thus this part of the application as filed does not provide basis for the "treatment of prostate cancer".

In the first case a "sufficient amount of phyto-oestrogen" is necessary to achieve treatment of prostate cancer and therefore this feature should be present in the claim, otherwise subject-matter is added through generalisation. In the second case only the reduction of the risk of development of cancer of the prostate is disclosed, which does not amount to treatment of prostate cancer.

According to **T296/96**:

"3.1 The content of a document must not be considered to be a reservoir from which features pertaining to separate embodiments could be combined in order to artificially create a particular embodiment. When assessing whether a feature has been disclosed in a document, the relevant question is whether a skilled person would seriously contemplate combining the different features cited in that document."

Decision **T1067/97** states it in a different way:

"2.1.3 According to established jurisprudence of the boards of appeal, if a claim is to be restricted to a preferred embodiment, it is normally not admissible under Article 123(2) EPC to extract isolated features from a set of features which have originally been disclosed in combination for that

embodiment. Such kind of amendment would only be justified in the absence of any clearly recognisable functional or structural relationship among said features."

In the present case it is evident that the patentee has isolated the "amelioration of prostate cancer" from the features in combination with which it was originally disclosed. The skilled person would consider that according to the application as filed (see first context), to achieve treatment of prostate cancer (therapeutic effect) a sufficient amount of phyto-oestrogen is necessary. That is what is directly and unambiguously derivable from the description. Thus the subject-matter defined in present claim 1 resulst in an undue generalisation and introduces subject-matter that was not present in the application as filed.

3.2 There is no basis for the feature "*for administration in unit dosage form*" of claim 1.

The feature "*for administration in unit dosage form*" was not present in the application as filed. Only original claim 7 mentions the unit dosage form, but referring back to a health supplement comprising a phyto-oestrogen selected from genistein, daidzein, biochanin A, and/or formononetin, and not to a medicament comprising an insoflavone phyto-oestrogen extract of soy or clover as defined in present claim 1. Therefore the unit dosage form was described in a particular embodiment or combination of features and the principles referred in the already mentioned **T296/96** and **T1067/97** apply to this situation as well.

The statement at the end of page 13 of the description:

"*The invention also concerns formulations containing the phyto-oestrogens discussed above together with a dietary suitable excipient, diluent, carrier, or with a food. Ideally the formulation is in the form of a pill, tablet, capsule, or similar dosage form.*"

or the first paragraph at page 14:

"...drinks, sterile injectable solutions, tablets, coated tablets, capsules, powders, drops, suspensions...or the like.The formulations may be in convenient dosage forms"

do not provide basis for the above feature either. It is established case law that originally undisclosed equivalents cannot be added by using a wider technical term in place of the single technical means originally disclosed (see Case Law of the Boards of Appeal of the EPO, 4th Ed. 2001, page 198 last paragraph, in particular **T265/88**). Hence, said amendment contradicts Article 123(2) EPC.

3.3 There is no basis for the amendments of the description.

It is submitted that during examination the patentee has substantially amended the description as originally filed, resulting in the addition of new subject-matter, contrary to art. 123(2) EPC.

Document **D17** is part of a transcript of a Patent Interference hearing at the USPTO and concerning the patent application US 08/910,837 (resulted in US 6,562,380) and deriving from the same PCT application as EP 0 656 786 B1. The purpose of a US interference procedure is to determine who was the first to invent when there are two patent applications or patents to cover the same subject. In this case the other patent under discussion was the US equivalent of WO 94/23716 (**D4**).

Voight is the attorney for G.E. Kelly (see page 2 of D17). In the transcripts at pages 53-55 the attorney for Kelly explains what was meant by "health supplement" in the international application PCT/AU93/00230 filed in Australia. Thus according to the patentee ***"Health supplement" is a term used in the field interchangeably with "dietary supplement"*** (see page 54, lines 1-5), and the later is according to him as defined by the FDA in the Dietary Supplement Health and Education Act of 1994 (see **D17**, page 55).

Document **D18** from the FDA web page gives the definition of "Dietary Supplement" as defined in the referred act (see first paragraph):

"*A dietary supplement is a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet....Dietary supplements are a special category under the general umbrella of "foods," not drugs".*

As such they are not intended to diagnose, treat, cure or prevent any disease. Thus the patentee recognizes that the term "Health Supplement" is not interchangeable with "Drug" or "Medicament".

In Europe the term used for this kind of products is "food supplement" and is defined in art. 2 of the Directive 2002/46/EC (document **D19**). It is clearly distinguished from a medicament, see art. 1.2 of the Directive:

"Article 1

1. This Directive concerns food supplements marketed as foodstuffs and presented as such. These products shall be delivered to the ultimate consumer only in a pre-packaged form.

2. This Directive shall not apply to medicinal products as defined by Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use (4).

Article 2

For the purposes of this Directive:

(a) 'food supplements' means foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities;..."

From the above it follows that according to the patentee a "health supplement" is different from, and does not overlap with, the term "drug" or "medicament".

Enclosed as document **D20** are pages 1, 8, 8a, 8b, 9-17 and 19 of the description with hand-written amendments, they were filed by the applicant on 23.12.2003. They are the same as those present in the Druckexemplar for grant. It should be noted that pages 8, 8a and 8b are previously filed pages to replace page 8. From these pages it can be seen that the amended description introduces several originally undisclosed embodiments:

- at page 8a, the last two paragraphs reproduce lines 4-16 of original page 8 that were describing a health supplement as the invention. This expression has been replaced by "medicament", when they are not equivalent according to the explanations given at the USPTO by the patentee.

- At page 13, lines 26-29, reference is made first to "the invention" which according to original page 8, lines 4-6 was a health supplement, and therefore it makes sense that the formulations decribed contain dietary suitable excipient or food. However, at line 29 the term formulation is replaced by "*the medicaments of the invention*" and the undisclosed word "*presented*" is introduced, with the absurd result that it now reads:

  "*The medicaments of the invention may be presented as nutricional supplements, pharmaceutical preparations, vitamin supplements, food additives or food supplemented with the specified active phyto-oestrogens of the invention...*"

  (Paragraph bridging pages 13-14 of the Druckexemplar, see also page 7, lines 23-24 of EP 0 656 786 B1).

The whole point here is that the application in 1993 was originally directed to Health Supplements (see title, description and originally filed claims), and a method of therapeutic treatment was only mentioned in the sidelines. Through successive amendments the European patent ended up several years later with claims directed to a medicament for 3 indications in the second medical use format. "Medicament" and "Health supplement" are not terms that can be used interchangeably, they are not equivalent. Therefore the amendment of the description of whole paragraphs that were referring to health supplements and that now refer to medicaments introduces subject matter which was not present in the application as filed.

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ABG Patentes S.L.

LIST OF DOCUMENTS CITED

D1: EP 0 656 786 B1

D2: WO 93/23069

D3: Australian Provisional Application PL2511 (19.05.1992) Priority Document

D4: WO 94/23716 (Tufts University School of Medicine)

D5: US 08/049,006 (filed 16.04.1993) priority document for WO94/23716

D6: Beckham, N. *Australian Wellbeing*, no. 29, 1988, pages 74-76: "Herbal Help to avoid Menopause Symptoms"

D7: Kaldas and Hughes *reproductive Toxicology Review* ,1989, 3(2), 81-89

D8: Wilcox et al. *BMJ*, vol. 301, 1990, pages 905-906 : "Oestrogenic effect of plant foods in postmenopausal women"

D9: Messina M., Barnes S., *J NCI*, 1991, pages 541-546 : "The role of Soy Products in Reducing Risk of Cancer"

D10: Adlercreutz et al. *Am J Clin Nutr*, 1991 54:1093-1100, "Urinary excretion of lignans and isoflavonoid phyto-oestrogens in Japanese men and women consuming a traditional Japanese diet"

D11: EP 135 172 A (Takeda)

D12: Eldridge et al. *J Agric Food Chem.* Pages 394-396, "Soybean isoflavones: Effect of Environment and Variety on composition"

D13: Statutory declaration of G.E. Kelly and GK1 (3.3.1997), filed 21.10.2003

D14: Knight et al, *Climacteric*, 1999, 2:79-84 "The effect of Promensil, an isoflavone extract, on menopausal symptoms"

D15: Van de Weijer, *Maturitas* 42 (2002) 187-193 : Isoflavones from red clover (Promensil) significantly reduce menopausal hot flush symptoms compared with placebo"

D16: Tice et al., *JAMA* July 2003, pages 207-213 : "Phytoestrogen Supplements for the treatment of Hot Flashes : the Isoflavone Clover Extract (ICE) Study"

D17: Transcripts of the Patent Interference hearing at the USPTO between G.E. Kelly vs S.L. Gorbach, B.R. Goldin and H. Adlercreutz (pages 1, 2-5, 46-57)

D18: Definition of a "Dietary Supplement" from the US Food and Drug Administration web page http://www.cfsan.fda.gov/~dms/ds-oview.html

D19: Official journal of The European communities, 12.7.2002, L183/51-57: Directive 2002/46/EC

D20: amended pages 1, 8, 8a, 8b, 9-17 and 19 of the description as filed by the applicant on 23.12.2003.

D1

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 656 786 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
15.09.2004 Bulletin 2004/38

(21) Application number: **93909679.8**

(22) Date of filing: **19.05.1993**

(51) Int Cl.[7]: **A61K 35/78**, A61P 15/12, A61P 35/00

(86) International application number:
PCT/AU1993/000230

(87) International publication number:
WO 1993/023069 (25.11.1993 Gazette 1993/28)

(54) USE OF ISOFLAVONE PHYTO-OESTROGEN EXTRACTS OF SOY OR CLOVER

VERWENDUNG VON ISOFLAVON PHYTO-ÖSTROGEN EXTRAKTEN VON SOJA ODER KLEE

UTILISATION D'EXTRAITS DES PHYTO-ESTROGENES D'ISOFLAVONES A PARTIR DE SOJA OU DE TREFLE

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **19.05.1992 AU 2511992**

(43) Date of publication of application:
14.06.1995 Bulletin 1995/24

(60) Divisional application:
04103669.0

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(56) References cited:

EP-A- 0 129 667
EP-A- 0 135 172
EP-A- 0 136 569
EP-A- 0 412 211
EP-A- 0 682 877
WO-A-96/10341
GB-A- 1 482 238
US-A- 5 352 384

- **PATENT ABSTRACTS OF JAPAN vol. 011, no. 096 (C-412), 26 March 1987 & JP 61 246124 A (YAMANOUCHI PHARMACEUT CO LTD), 1 November 1986, & DATABASE WPI Week 8650 Derwent Publications Ltd., London, GB; AN 86-329435 & JP 61 246 124 (YAMANOUCHI PHARM CO LTD) , 1 November 1986**
- **PATENT ABSTRACTS OF JAPAN vol. 014, no. 246 (C-0722), 25 May 1990 & JP 02 067218 A (NAGAKURA SEIYAKU KK), 7 March 1990,**
- **PATENT ABSTRACTS OF JAPAN, C-757, page 17; & JP,A,02 160 722 (NIPPON OIL AND FATS CO LTD) 20 June 1990 (20.06.90), see entire Abstract.**
- **PATENT ABSTRACTS OF JAPAN, C-452, page 118; & JP,A,62 106 016 (YAMANOUCHI PHARMACEUT CO. LTD) 16 May 1987 (16.05.87), see entire Abstract.**
- **PATENT ABSTRACTS OF JAPAN, C-722, page 98; & JP,A,02 067 218 (NAGAKURA SEIYAKU K.K.) 7 March 1990 (07-03-90), see entire Abstract, particularly formula I.**
- **CHEMICAL ABSTRACTS, Volume 115, No. 8, issued 26 August 1991 (26.08.91) (Columbus, Ohio) USA, LIU, Y. et al., "Effects of Solid Dispersion of Daidzein on the Blood Pressure of Spontaneously Hypertensive Rats", page 406, Abstract No. 78763p, Shenyang Yaoxueyuan Xuebao 1991, 8(2) 105-8 (Ch.), see entire Abstract.**

EP 0 656 786 B1

Printed by Jouve, 75001 PARIS (FR)

Description

TECHNICAL FIELD

[0001] This invention relates to medicaments containing phyto-oestrogens, or phyto-oestrogen metabolites used to treat pre-menstrual syndrome, menopausal symptoms, or prostate cancer.

BACKGROUND ART

[0002] The medicaments for use in accordance with the invention are made from extracts of certain plants with the particular purpose of enrichment for phyto-oestrogens, both in their natural state and their closely related derivatives and metabolites.

[0003] Plants which are used as foodstuffs or medicinal herbs contain a wide variety of chemicals which are assimilated into the body following ingestion. Some of these chemicals are important nutrients for man and animals (eg. fats, carbohydrates, proteins, vitamins, minerals) while others have none, or little or no known nutritional value. The phyto-oestrogens hitherto have fallen into this latter category of no known nutritional value.

[0004] There are 3 principal classes of phyto-oestrogens, viz. isoflavones, lignans, and coumestans. The isoflavones are thought to have a broad range of biological functions in plants, although these are poorly understood. However, two particular functions are recognised - (a) as phyto-alexin or stressor chemicals which are secreted by the plant in response to attack by parasites such as insects, fungi, viruses, etc and which display activity against these parasites, and (b) chemicals which encourage colonisation of nitrogen-fixing bacteria on the roots of legumes.
The biological functions in plants of the lignans and coumestans is not generally understood.

[0005] The different types of phyto-oestrogens are as follows.

Type 1 phyto-oestrogens - (isoflavones)

[0006] Isoflavones appear to be widely distributed in the plant kingdom and over 700 different isoflavones are described. However, the isoflavones which display oestrogenic activity belong to a small sub-group and are restricted almost exclusively to the *Leguminosae* family. The known oestrogenic isoflavones are daidzein, formononetin, genistein and biochanin A. In common human foodstuffs such as soya, chickpeas, lentils and beans, the total levels of the oestrogenic isoflavones range between about 40-and 300 mg per 100 g dry weight.

[0007] In the raw plant material, isoflavones occur principally as glycosides. Following ingestion by man and animals, the glycoside moiety is hydrolysed free by a combination of gastric acid hydrolysis and fermentation by intestinal bacteria. Some of the isoflavones in the aglucone form are absorbed directly and circulate in the blood, while the remainder are metabolised by intestinal fermentation to a variety of compounds which are also absorbed. The absorbed isoflavones and their metabolites appear to undergo little or no further metabolism in the body, being readily transported in the bloodstream, and ultimately being excreted in the urine.

Type 2 phyto-oestrogens (lignans).

[0008] Lignans are widely distributed in the plant kingdom. Over one hundred lignans are described and they are reported in common human foodstuffs such as cereals, fruits and vegetables. Oilseeds such as flax (linseed) have the highest known levels at 20-60 mg/100 g dry weight, while cereals and legumes have much lower levels at 0.3-0.6 mg/100 g, and vegetables even lower levels at 0.1-0.2 mg/100 g. The most common lignan described is metairesinol. Dietary lignans also appear to be metabolised fairly efficiently within the gut by bacterial fermentation, yielding metabolites such as enterodiol and enterolactone which are absorbed into- the bloodstream and excreted in the urine.

Type 3 phyto-oestrogens (coumestans).

[0009] Compared to isoflavones and lignans, oestrogenic coumestans appear to have a relatively restricted distribution in plants and generally occur at much lower levels. Alfalfa, ladino clover and some other fodder crops such as barrel medic may have significant levels and have been reported to cause reproductive dysfunction in grazing animals. In the human diet, the important sources of coumestans are sprouts of soya and alfalfa where levels up to 7 mg/100g dry weight are reported. Whole soyabeans and other common foodstuff legumes contain levels of approx. 0.12 mg/100 g dry weight and most of that is concentrated in the seed hull which commonly is removed in the preparation of human foodstuffs.

Type 4 phyto-oestrogens (oestrogens).

[0010] These are compounds closely related to animal oestrogens such as oestrone, oestradiol and oestriol. These have been described in plants such as liquorice, apple, French bean, pomegranate and date palm. Little is known of the metabolism and biological significance of these chemicals in humans and animals.

[0011] The full range of biological effects in animals of these dietary phyto-oestrogens has received only recent study. A primary effect appears to be associated with their close structural relationship to naturally-occurring oestrogens which allows the phyto-oestrogens to mimic the effects of the endogenous oestrogens. The known biological effects of phyto-oestrogens can be summarised thus:

In vitro

(a) bind to both cytoplasmic and nuclear membrane (Type II) oestrogen receptors on human tissues;
(b) strongly compete with oestrogens for oestrogen receptors, but only weakly stimulate those receptors;
(c) strongly stimulate the production of sex hormone-binding globulin (SHBG) from human cells;

In vivo

(d) weakly oestrogenic in animals;
(e) competitively-inhibit the response of tissue to oestrogens.

[0012] The three major types of phyto-oestrogens appear to act at the cellular level in a similar manner, that is through interaction with cell surface oestrogen receptors. In the body, naturally-occurring oestrogens circulating in the blood largely exert their activity by interaction with oestrogen receptors on cell surfaces; such interactions then triggering a particular biological function of that particular cell. Phyto-oestrogens are able to bind to those oestrogen receptors because the structure of these compounds so closely resembles the endogenous oestrogens, but unlike the animal oestrogens, phyto-oestrogens only weakly activate the oestrogen receptor.

[0013] As a result of phyto-oestrogens and endogenous oestrogens competing for the oestrogen-binding sites on cells, the more weakly oestrogenic phyto-oestrogens can be considered to have an anti-oestrogenic effect. This phenomenon is known as competitive-inhibition, by which is meant that the biological effect of an active substance is impaired by the competitive binding to a target receptor of a similar but less active compound.

[0014] Thus a primary biological effect of phyto-oestrogens is held to be competitive inhibition of endogenous oestrogens. However, another more direct effect is the stimulation of synthesis of SHBG in the liver, as occurs with orally administered synthetic steroidal oestrogens. High levels of dietary phyto-oestrogens are thought to be responsible for the higher SHBG levels seen in vegetarians and in cultures maintaining traditional (high legume-containing) diets.

[0015] At high levels, dietary phyto-oestrogens can have profound physiological effects. An example of this is sheep and cattle grazing pastures containing a high proportion of subterranean clover or red clover which can contain levels of phyto-oestrogens as high as 5% of the dry weight of the plant. As a result of the competitively-inhibitory effect of the dietary phyto-oestrogens on endogenous oestrogen function in the hypothalamus, male and female sheep and cows can develop androgenic symptoms.

[0016] Such high dietary levels of phyto-oestrogens, however, are rare. It is far more common that most animal and human diets contain low to moderate levels of phyto-oestrogens, and there is growing epidemiological evidence that such levels have a beneficial effect on human health.

[0017] In most traditional human diets in developing countries, the principal phyto-oestrogens consumed are isoflavones because of the generally high reliance on legumes (also known as pulses) as a source of protein. The general consumption rates (g/day/person) for legumes for different regions currently are approximately: Japan (50-90), India (40-80), South America (30-70), North Africa (40-50), Central/Southern Africa (20-50) and Southern Mediterranean (30-60). Legumes also are a source of lignans and, to a much lesser extent, coumestans, and the additional cereal and vegetables in the diet would also boost the lignan intake. However, the isoflavone intake in these traditional cultures with high legume consumption would typically be much in excess of either lignan or coumestan intake.

[0018] The major types of legumes used in traditional diets include soya, chickpeas, lentils, ground nuts, beans (e.g. broad, haricot, kidney, lima, navy), and grams (bengal, horse and green).

[0019] In Western, developed countries, the daily intake of dietary phyto-oestrogens generally is negligible to low. In Western Europe, North America and Australasia, legumes were a major source of protein for the majority of the populations up to the end of the 19th century. From that time, legume consumption has declined significantly, being replaced in the diet with protein of animal origin. Average legume consumption in these regions currently is between 5-15 g/day/person with a significant proportion of the population ingesting little to no legumes or other phyto-oestrogen containing foods on a regular basis. Moreover, the types of legumes consumed in these regions (e.g. garden peas, French beans) have a typically lower isoflavone content than legumes such as soya and chick peas.

[0020] Based on typical consumption rates and types of foodstuffs consumed, the typical phyto-oestrogen intake (mg/day) for different regions can be calculated approximately as

	Isoflavones	Lignans	Coumestans
Japan	50-300	2-5	0.5
Australia	2-25	1-5	0.2

[0021] Thus it can be seen that regions which have maintained traditional diets have a higher average daily intake of phyto-oestrogens, particularly isoflavones, compared to western countries. People in communities such as Japan or developing countries with high legume intake excrete substantially higher phyto-oestrogen metabolites in their urine compared to people in Western countries. Within the latter, vegetarians also excrete higher phyto-oestrogen metabolite levels than do those consuming a more typical, omnivorous Western diet.

[0022] The presence of relatively large amounts of phyto-oestrogen metabolites in urine serves to highlight their potential biological significance. It has been shown that total urinary excretion of isoflavones and their active metabolites in people consuming moderate amounts of legumes is greatly in excess (up to 10,000 x) of steroidal oestrogen levels. So that while the oestrogenicity of isoflavones to oestrogen receptors is only about 1% that of endogenous oestrogens, this weaker effect is off-set by the much higher blood levels of the isoflavones.

[0023] It is known that legumes have formed an important part of the human diet over the past 20,000-30,000 years. It therefore follows that human metabolism has evolved over at least this period in the presence of relatively large levels of dietary phyto-oestrogens, particularly isoflavones. Given the known biological effects of phyto-oestrogens, it also follows that endogenous oestrogen metabolism and function has evolved in the face of significant competitive inhibiting effects of phyto-oestrogens. It has been speculated that the presence of significant dietary levels of phyto-oestrogens in recent human evolution has led to a degree of adaption by tissues responsive to reproductive hormones to these dietary components. That is, both the rate of production and/or the function of endogenous oestrogens may be either dependent upon or influenced by the presence of phyto-oestrogens in the body. It follows therefore that a relative deficiency of dietary phyto-oestrogens could be expected to lead to an imbalance of endogenous oestrogen metabolism.

[0024] There is increasing interest in the likely contribution of a relative deficiency of dietary phyto-oestrogens to the development of the so-called "Western diseases", especially cancer of the breast, benign (cystic) breast disease, cancer of the uterus, cancer of the prostate, cancer of the bowel, pre-menstrual syndrome, menopausal syndrome, and atherosclerosis. All of these diseases are associated to a greater or lesser extent to oestrogen metabolism, and oestrogen function is either known or is suspected to play a role in their aetiology and/or pathogenesis.

[0025] Each of these diseases occurs at much higher incidence in Western, developed countries than it does in developing communities. Moreover, it is thought that in Western communities, the incidences of each have risen over the past century. It is also generally held, that of all the environmental factors likely to be contributing to this phenomenon, diet is the principal factor. Of those dietary components with the potential to influence the aetiology of oestrogen-related disease, there is a growing awareness that phyto-oestrogens may have important potential.

[0026] The beneficial effects of phyto-oestrogens on human health are thought to derive from at least two principal function, those being (i) competitive-inhibition of the function of endogenous oestrogens, and (ii) the stimulation of production of SHBG. SHBG plays an important role in primates in binding and transporting the reproductive hormones (oestrogens, androgens) in blood so that the availability of reproductive hormones is regulated to a large degree by SHBG levels. Higher SHBG levels are considered beneficial in leading to a reduction in both blood levels of unbound (and unregulated) reproductive hormones and metabolic clearance rates of the hormones. Although isoflavones are potent stimulators of SHBG synthesis, they only weakly bind to SHBG, so that the increased SHBG levels resulting from the dietary isoflavones are largely available for binding to endogenous oestrogens.

[0027] In terms of directly identifying the beneficial effects of phyto-oestrogens in amelioration of any or all of the "Western diseases", there are only two examples. In one example, the diets of women, with menopausal syndrome were supplemented with foodstuffs (soya, linseed, red clover) high in phyto-oestrogens, and an alleviation of menopausal symptoms to an extent similar to that obtained with replacement therapy with synthetic oestrogens was achieved; that effect was ascribed to the phyto-oestrogen content of the supplement. In the other example, legumes such as soya and various pulses have been shown to have a hypocholesterolaemic effect in humans; this effect has not been ascribed to phyto-oestrogens, although purified isoflavones do have a hypocholesterolaemic effect in animals with artificially-induced hypercholesterolaemia.

[0028] In summary, it could reasonably be deduced that the inclusion of greater levels of foodstuffs high in phyto-oestrogens in the standard diets of men and women in developed countries could be expected to redress a general imbalance of endogenous reproductive hormone metabolism, thereby reducing the predisposition of those communities to the above diseases. While there are various types of phyto-oestrogens which may be suitable to this end, the large

discrepancy in isoflavone consumption between communities with Western and traditional diets suggest that foodstuffs with high isoflavone content are of prime interest.

[0029] However it is unrealistic to expect that public education programmes would readily convert communities in developed countries from a diet where the protein content is predominantly animal-derived, to one where the protein is predominantly legume-derived. Moreover, the legumes which are commonly consumed in developed countries are relatively poor sources of phyto-oestrogens and the general acceptance in the community of less well-known legumes with higher phyto-oestrogen content would be necessarily a slow process. Also, the highly variable levels of phyto-oestrogens in foodstuffs relating to plant strain type, degree of plant maturity, and climatic and other environmental conditions suggests that the supply of an assured amount of phyto-oestrogens through the use of whole foodstuffs may be difficult.

[0030] An alternative strategy is to make available either (i) phyto-oestrogens in a purified form, or (ii) foodstuffs which are enriched for phyto-oestrogens. In this way, the phyto-oestrogen could be added to the diet in a convenient form as a supplement without requiring any substantive change to the diet.

[0031] The present invention provides the use of an isoflavone phyto-oestrogen extract of soy or clover for the manufacture of a medicament for administration in unit dosage form for the treatment of pre-menstrual syndrome, symptoms associated with the menopause, or prostate cancer.

[0032] The medicament may further comprise at least one dietary suitable excipient, preferably wherein the medicament is in the form of a tablet or capsule.

[0033] The isoflavone phyto-estrogens are preferably present in an amount of from about 20mg to 200mg per dosage unit, optionally wherein the amount is 50mg to 150mg.

[0034] In preferred embodiments the extract is obtained from clover or soybean. The extract is most preferably obtained from the leaves of clover or the hypocotyls of soybean.

[0035] The extract is preferably in liquid form, more preferably an aqueous organic solvent extract, even more preferably an aqueous alcohol extract.

[0036] In preferred embodiments the administration of the medicament is at least daily over a period of at least a month.

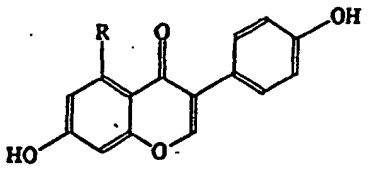
[0037] The medicaments of the present invention are specifically enriched for isoflavones selected from genistein, daidzein, formononetin and biochanin A, or their natural glycoside form, or their analogues.

[0038] Preferably the medicament contains an excipient, a diluent, a carrier or the like, or else it may be is mixed with food or can be consumed directly. It is also preferred that the ratio of genistein and/or it methylated derivative biochanin A to daidzein and/or its methylated derivative formononetin is between 1:2 to 2:1. Other plant components with oestrogenic activity including lignans, coumestans and flavones may also be present in extract, but it is held that these are of secondary importance to the predominant isoflavones. The term phyto-estrogens is used hereafter to indicate a predominance of isoflavones with lesser amounts of lignans, coumestans and flavones.

[0039] The medicaments in accordance with the invention can be used to improve the health of a human by administering to the human regularly on a daily basis over a sufficient period such as at least a month. The health conditions which are ameliorated are cancer of the prostate pre-menstrual syndrome (also known as pre-menstrual tension), or adverse symptoms associated with menopause in women.

[0040] The phyto-oestrogens are extracted from a clover, such as red clover or subterranean clover, or from soya which contain high levels of phyto-oestrogens.

[0041] Various different isoflavones have been identified from these sources - they are principally genistein, biochanin A, daidzein, formononetin and glycitein. In plants these compounds occur principally in a glycoside form bound to sugars such as glucose, with smaller amounts present as the aglucone forms. The formulae of the isoflavones are:



daidzein R = H
genistein R = OH

[0042] The structure of biochanin A is the same as for genistein but with a 4'-methoxy group, and similarly formononetin has the same structure as daidzein, but with a 4'-methoxy group.

[0043] Following ingestion by humans, the glycosidic isoflavones are hydrolysed to the aglucone form and biochanin A and formononetin are demethylated by bacterial fermentation to genistein and daidzein respectively. A small pro-

portion of these free isoflavones are absorbed directly from the bowel and circulate in the blood. The bulk of the isoflavones, however, remain in the bowel and undergo fermentation to form various metabolites which also are absorbed into the bloodstream. The principal metabolites which have been identified are equol and O-*des*methylangolensin.

[0044] *In vitro* and *in vivo* studies have indicated that genistein, biochanin A, equol, daidzein, formononetin all have oestrogenic activity in descending order. O-*des*methylangolensin is only very weakly oestrogenic and glycitein is non-oestrogenic.

[0045] In animal and *in vitro* studies, genistein has been shown to have greater oestrogenic/anti-oestrogenic activity and SHBG-stimulating capacity than the other isoflavones or their metabolites (approximately 10 times that of daidzein and formononetin). However, the full range of biological effects of the different isoflavones have yet to be fully determined, and in particular their relative efficacies in the different biological effects such as oestrogenicity, hypocholesterolaemia, anti-angiogenesis, anti-oxidation, anti-carcinogenesis for example are not yet fully known.

[0046] It is thought that because the methyl forms (biochanin A and formononetin) ultimately are largely demethylated to their principals, genistein and daidzein with improved biological efficacy, then it is unimportant whether the isoflavones are present in accordance with the claimed invention in the methylated or demethylated forms.

[0047] Given that the relative biological importance of the two isoflavone groups (being genistein and daidzein) to human health remains unclear, and that each might indeed have different importance, plus the fact that both isoflavones are present in the diet in approximately equal proportions, then it is prudent that both isoflavones be present in accordance with the claimed invention in approximately equal proportions.

[0048] The ideal soy or clover sources of phyto-oestrogens in accordance with the invention are preferably those which (i) are readily available, (ii) are relatively inexpensive, (iii) are readily and economically processed so as to yield the extract, (iv) have a high isoflavone content so as to provide high yields, and (v) have no known toxic components requiring selective removal or inactivation.

[0049] Certain clovers, such as red clover (*T. pratense*) and subterranean clover *(T. subterranean)* are the preferred sources. On a dry weight basis, these clovers contain the highest amounts of oestrogenic isoflavones of all legumes tested to date with levels of 3-5 *g%(T- subterranean)* and 1-3 g% (*T. pratense*). In comparison, soya flour has a level of 0.15-0.30 g%, lentils (0.08-0.12 g%), chick peas (0.07-0.13 g%), and garden peas (0.02-0.03 g%). Thus it can be seen that clovers contain approximately at least 10-30 times by weight the isoflavone content of other commonly available, human leguminous foodstuffs meaning that for manufacturing purposes, the yield of isoflavones per unit weight of plant material is many times greater from clover than from other legumes.

[0050] Red clover and subterranean clover also are common fodder crops and are readily grown and are widely available. Clovers also are comparatively cheaper ($200/tonne) than crops such as soya and lentils ($500/tonne).

[0051] With clovers, the isoflavones are recovered from the leaf rather than from the seed in the case of soya, beans, nuts and grams. This provides a substantially higher yield of isoflavones per unit area of pasture for clovers compared to other legumes because of the greater leaf matter compared to seed matter recovered per plant.

[0052] Clovers also have an extended growing season, and faster growth rates compared to those legumes such as soya, lentils or chick peas where the seed is the end-product Clover can be cropped for its leaf content repeatedly over a single growing season. An additional benefit of this is that as phyto-alexins, the isoflavone content increases in response to the stress of cropping.

[0053] Thus it can be seen that in clovers versus other legumes provide a combination of (a) higher isoflavone content per dry weight of plant, (b) a higher yield of dry matter containing isoflavones per plant, and (c) a higher yield of dry matter per hectare.

[0054] An additional feature of clovers is that there are wide varieties of cultivars with widely differing isoflavone levels and types. This allows blending of different cultivars to achieve the desired ratio of the different isoflavones, although it is equally possible to use a single cultivar which provides the desired ratio.

[0055] Soyabean flour may be used as the source of phyto-oestrogens but the substantial poorer (approx. 10%) yield of isoflavones compared to clovers means that the manufacturing costs are substantially greater and there is substantially greater amounts of waste products which requires disposal or further treatment for re-use as a foodstuff. An alternative, however, to the use of whole soya for this purpose, is to use the hull and hypocotyl (or germ) of the whole soyabean. The hull and hypocotyl represent only a small proportion by weight (8% and 2% respectively) of the intact bean. However, the coumestrol content of soya is concentrated in the hull, and the daidzein content of soya is concentrated in the hypocotyl. The two cotyledons which comprise the bulk of the soyabean (90% by weight) contain the bulk of the genistein content of soya. During standard processing of soyabeans, the hulls being a fibrous component with little or no perceived nutritional value normally are separated and removed by physical means. The hypocotyls become separated following the splitting of the cotyledons, and while these currently generally are not deliberately isolated, they may be separated and isolated by passing the disturbed soyabeans over a sieve of sufficient pore size to selectively remove the small hypocotyl. The hypocotyl contains approx. 1.0-1.5 g% isoflavones (95% daidzein, 5% genistein). The raw hypocotyl and hull material can be ground or milled to produce, for example, a dry powder or flour which then could be either blended or used separately as a dietary supplement in a variety of ways including, for

example, as a powder, in a liquid form, in a granulated form, in a tablet or encapsulated form, or added to other prepared foodstuffs. For use in accordance with the invention it is further processed to yield an enriched extract of phyto-oestrogens. This material also could be added to clover extract in accordance with the invention.

[0056] In plants, the oestrogenic isoflavones are restricted principally to the leaf, fruit and root; the stem and petiole contain very little. With soya crops, the leaves are rarely regarded as foodstuff; indeed with these crops, the plants normally are allowed to die and dry out before the seed crop is harvested. Nevertheless, the fresh leaves of these crops could be regarded as a source of phyto-oestrogens for the invention although the much lower isoflavone content of the leaves of these crops compared to clovers, plus their generally slow growth compared to clovers, suggests that they would not be a preferred source of large-scale isoflavone enrichment.

[0057] To provide a similar amount of isoflavone to that contained in most traditional legume-rich diets (50-100 mg oestrogenic isoflavones/day) would require an average daily consumption of 3-6 g dry weight or 15-30 g wet weight of specially selected cultivars of clover with particularly high isoflavone levels. Clover grasses generally are not eaten by humans, except to a limited extent as sprouts of some of the pleasanter tasting varieties. Isoflavones are intensely astringent and are responsible in large part for the bitter taste of legumes. Thus the types of bean sprouts, clover sprouts and alfalfa sprouts generally available have been selected on the basis of cultivar and of age for pleasant taste, and in so doing inadvertently have been selected for low isoflavone content. Of the sprouts currently available in Western countries for human consumption, between approx. 100-250 g would need to be consumed daily to provide a dosage of 50-100 mg isoflavones. Certainty clovers and other legume sprouts are not generally eaten in such sufficient quantities by humans to obtain the advantages of the present invention.

[0058] The invention also concerns formulations containing the phyto-oestrogens discussed above together with a dietary suitable excipient, diluent, carrier, or with a food Ideally the formulation is in the form of a pill, tablet, capsule, or similar dosage form.

[0059] The medicaments of the invention may be presented as nutritional supplements, pharmaceutical preparations, vitamin supplements, food additives or foods supplemented with the specified active phyto-oestrogens of the invention, liquid or solid preparations, including drinks, sterile injectable solutions, tablets, coated tablets, capsules, powders, drops, suspensions, or syrups, ointments, lotions, creams, pastes, gels, or the like. The formulations may be in convenient dosage forms, and may also include other active ingredients, and/or may contain conventional excipients, carriers and diluents. The medicaments are preferably presented as herbal remedies and treatments.

[0060] The invention is directed to the treatment of the specified conditions using the specified phyto-oestrogen medicaments. The preferred amounts to be administered to the human fall within 20 - 200 mg on a daily basis. More preferably the dosage is from 50 - 150 mg on a daily basis, and most preferably at a dosage of about 100 mg. If desired greater dosages can be administered for therapeutic reasons. In contrast to prior practices such high dosages were not possible. For example, dosages of up to or greater than 1000 mg may be suitable. In order to obtain the benefits of the invention, the treatment with the isoflavones should continue for a considerable period, ideally for at least a month, and ideally continuously for the whole period for which the health improvement advantages should accrue.

[0061] The medicament according to the claimed use of the present invention yields a constant and accurately known amount of isoflavones. The product is also ideally a natural product, which has advantages for consumer acceptance, and in accordance with the supposed theory behind the invention may very possibly be one of the main causes for its beneficial effects. Whole legumes have a widely variable isoflavone content due to two main causes: the type of legume and the environmental effect. The type of legume typically has a wide range of isoflavone content. The miligram of isoflavone per hundred gram of whole foodstuff (dry weight) is given in the following table:

Soya Products	
- Whole Soya	150-300
- Soya Milk	25 - 40 (mg per 200 ml)
- Tofu	55 - 95
Lentils	80 - 120
Chickpeas	70- 130
Broad beans	15 - 20
Garden peas	15 -25

[0062] Thus common leguminous foodstuffs consumed in Western countries (broad beans, garden peas etc) have relatively low oestrogenic isoflavone content and exceptionally large amounts of these would need to be consumed daily to approximate those isoflavone levels consumed in traditional diets. Most Western cultures do not traditionally eat legumes with high isoflavone contents, and those soya products (milk, tofu etc) which are becoming increasingly popular in Western countries, also have relatively low isoflavone levels compared to whole soya, indicating that rela-

tively large amounts of these would need to be consumed on a regular basis to deliver the required isoflavone levels.

[0063] The environmental effect arises because the isoflavone levels in any species of plant depend greatly on the age of the plant, the climatic conditions where it is grown, the fertiliser and so forth. Therefore constant and consistant dosage is very difficult with ordinary whole foodstuffs. The accurately determined quality and quantity of the active isoflavones in the medicament, and its easy consumability when compared with the almost impossible task of eating huge amounts of often practically inedible foods, is therefore an import feature of the invention, for helping in overcoming the specified health problems.

[0064] The health problems, include the treatment of PMS and menopausal symptoms. The invention therefore is directed to the use of the specified medicament for the treatment of a human, to combat the specified conditions associated with phyto-oestrogen deficiency, which comprises administering to the human an effective amount of phyto-oestrogen principally isoflavone but which might also include relatively smaller amounts of lignans and coumestans, ideally in a concentrated form, wherein the isoflavones include genistein, and/or biochanin A, and/or daidzein, and/or formononetin.

[0065] Cancer of the prostate generally is considered to be associated with sex hormone dysfunction and the growth of prostatic cancer cells is influenced by oestrogens and androgens. The incidence of prostatic cancer is low in communities with high legume intake and, conversely, is high in Western socieites. Phyto-oestrogens are though to protect from development of prostatic cancer. One mechanism may be the effect of phyto-oestrogens on lowering the proportion of unbound:bound reproductive hormones in the blood. However, there is other evidence to suggest that phyto-oestrogens, particularly isoflavones, can have a direct influence on certain cellular enzymes within prostatic cells.

[0066] Pre-menstrual syndrome has uncertain aetiology and pathogenesis, although most certainly is associated with reproductive hormone dysfunction. It also is a syndrome which has reportedly lower incidence in communities maintaining traditional high-legume diets. It is proposed that phyto-oestrogens will alleviate this condition by restoring balance to oestrogen metabolism.

[0067] Menopausal syndrome is associated with changes in the oestrogen profile in the body with advancing age. Adverse clinical symptoms may be treated with oestrogen replacement therapy. There is evidence that foodstuffs high in phyto-oestrogens are a suitable alternative to synthetic hormones in this respect, producing alleviation of adverse clinical symptoms. Again, it is proposed that phyto-oestrogens will function by restoring balance to oestrogen metabolism.

MODES FOR CARRYING OUT THE INVENTION

[0068] The invention is now described with reference to various examples.

EXAMPLE 1 - Preparation of Red Clover Product

[0069] Tablets were prepared using red clover in accordance with the following procedure. The raw plant material is harvested and dried; such drying being either sun-drying or from applied heat.

[0070] The dried product is then preferably chaffed, before the following extraction step, although this can be omitted if desired.

[0071] The dried material is extracted in an aqueous: organic solvent mix. The aqueous phase is required to extract the water-soluble glycoside form of isoflavones, while the organic solvent is required to solubilise the water-insoluble aglycone form. The organic solvent can be either alcohol, chloroform, acetone or ethyl acetate. The ratio of solvent in the water can be between 0.1 % and 99.9%. The preferred method is to use 60% alcohol in water.

[0072] The isoflavones are extracted by exposing the plant material to the water:solvent mix. The exposure time in general terms is indirectly proportional to the temperature of the mixture. The temperature of the mix can range between ambient temperature and boiling temperature. The exposure time can be between 1 hour and 4 weeks or even longer. It has been determined that the adequate times for maximal recovery of isoflavones are 2 weeks at 50°C and 24 hours at 90°C. The supernatant is separated from the undissolved plant material and the organic solvent removed by distillation The aqueous supernatant then is concentrated, typically by distillation.

[0073] Additional processing steps can be used, if desired, to convert the extracted natural product to capsule, tablet, or other convenient form for ingestion, using normal techniques for doing this. Otherwise the product can be packaged as a convenient food additive.

EXAMPLE 2 - preparation of soya hypocotyl product.

[0074] Soyabeans were heated in dry air so that the hull became brittle. The beans then were processed through a tumble mill which removed the hull and split the bean the two cotyledons and the small-sized hypocotyl which separated from each other. The light-weight hulls then were removed by an air stream. The small-sized hypocotyls were separated

from the larger cotyledons by sieving through a steel wire mesh with apertures of 1 mm x 1 mm. This yielded approximately 87% purity of hypocotyls with 13% contamination by small cotyledon chips.

[0075] Normal soybean processing steps isolate the hulls and then these are discarded or processed separately for use in human and animal foodstuffs. The hypocotyls normally are not separated and are processed along with the cotyledons. However, a small number of soybean processors are separating hypocotyls by the above methods in order to reduce the astringent taste of soyflour for human consumption, and currently these hypocotyls are either discarded or processed to flour for use in animal feed.

EXAMPLE 3 - effect of administering red clover extract to humans (not of the invention)

[0076] Seven normal individuals were studied for the comparative effects of red clover extract and whole legumes on blood cholesterol levels. All the individuals were consuming a standard Western diet with minimal levels of legumes.

[0077] Three men consumed between 100-150 g haricot or navy beans daily for 3 weeks as a supplement to their normal diet. This yielded an approximate daily isoflavone dosage or between 60-100 mg.

[0078] Four other individuals (3 men, 1 woman) consumed 5 g of red clover extract containing 100 mg isoflavones daily for 3 weeks.

[0079] Total serum cholesterol levels were determined immediately before and immediately following the challenge.

	Pre-treatment	Post-treatment	% change
Beans only			
Patient 1	5.77	5.46	- 5.4
Patient 2	6.24	6.12	- 1.9
Patient 3	7.45	8.51	+14.3
Red clover extract			
Patient 5	6.53	5.90	- 9.6
Patient 6	7.43	6.63	-10.8
Patient 7	6.33	5.50	-13.1
Patient 8	6.98	7.28	+ 4.3

[0080] The red clover extract had a significantly ($P < 0.05$) greater hypocholesterolaemic effect than did the whole beans.

[0081] Neither of the treatments produced any untoward side effects, although the whole bean eaters reported greater difficulty with compliance of treatment than did those taking the red clover extract.

EXAMPLE 4 - effect of administering soy hypocotyls to humans

[0082] Fifteen volunteers (8 women, 7 men) were given 5 g of soy hypocotyl containing (45 mg daidzein and 5 mg genistein) daily for 2 months. The hypocotyl was consumed as a powder added to the diet.

[0083] The effects on cholesterol levels are shown in the following table. The individuals are grouped according to their pre-treatment cholesterol levels (high, medium, low).

		Range (mean) unmol/L	
	n	Pre-treatment	Post-treatment
Group 1	6	6.3 - 8.4 (7.1)	5.4 - 6.5 (6.1)
Group 2	6	5.0 - 6.2 (5.5)	4.7 - 5.9 (5.1)
Group 3	3	3.3- 4.7 (4.2)	3.4 - 4.6 (4.1)

[0084] The results show a significant fall in total cholesterol levels in those individuals with cholesterol levels considered to be at the upper end of the normal range.

[0085] In addition, 1 woman reported substantial amelioration of her benign breast disease problem associated with mid-cycle swelling and tenderness, and another woman reported regularisation of her menstrual cycle and reduced menstrual bleeding. Both of these effects were regarded as beneficial.

[0086] No other side-effects were reported as a result of the treatment.

Claims

1. The use of an isoflavone phyto-oestrogen extract of soy or clover, for the manufacture of a medicament for administration in unit dosage form for the treatment of pre-menstrual syndrome, symptoms associated with menopause, or prostate cancer.

2. A use as claimed in claim 1, wherein the medicament further comprises at least one dietary suitable excipient.

3. A use as claimed in claim 1 or 2, wherein the isoflavone phyto-oestrogen is extracted from soya.

4. A use as claimed in claim 3, wherein the isoflavone phyto-oestrogen is extracted from soya hypocotyls.

5. A use as claimed in claim 1 or 2, wherein the isoflavone phyto-oestrogen is extracted from dover.

6. A use as claimed in any one of claims 1 to 4, wherein the isoflavone phyto-oestrogen extract comprises one or more of genistein, daidzein, or glycosides thereof, or metabolites or derivatives thereof.

7. A use as claimed in claim 1 or claim 2, wherein the isoflavone phyto-oestrogen comprises genistein and/or biochanin A:daidzein and/or formononetin, present in a ratio of from about 1:2 to 2:1.

8. A use as claimed in any of claims 1 to 7, wherein the isoflavone phyto-oestrogens are present in an amount from about 20 mg to 200 mg per unit dose, optionally where the amount is 50 mg to 150 mg.

9. A use as claimed in any preceding claim, wherein administration of the medicament is administered at least daily over a period of at least a month.

10. A use as claimed in any preceding claim, wherein the extract includes coumestans, lignans and flavones.

11. A use as claimed in any preceding claim, wherein the unit dosage form is a tablet or capsule.

Patentansprüche

1. Verwendung eines Isoflavon-Phytoöstrogen-Extrakts von Soja oder Klee für die Herstellung eines Medikaments zur Verabreichung in Dosierungseinheitsform für die Behandlung des prämenstruellen Syndroms, von Symptomen, die mit der Menopause verbunden sind, oder von Prostatakrebs.

2. Verwendung wie in Anspruch 1 beansprucht, wobei das Medikament außerdem wenigstens ein nahrungsmittelgeeignetes Excipiens umfasst.

3. Verwendung wie in Anspruch 1 oder 2 beansprucht, wobei das Isoflavon-Phytoöstrogen aus Soja extrahiert wird.

4. Verwendung wie in Anspruch 3 beansprucht, wobei das Isoflavon-Phytoöstrogen aus Sojahypokotylen extrahiert wird.

5. Verwendung wie in Anspruch 1 oder 2 beansprucht, wobei das Isoflavon-Phytoöstrogen aus Klee extrahiert wird.

6. Verwendung wie in einem der Ansprüche 1 bis 4 beansprucht, wobei der Isoflavon-Phytoöstrogen-Extrakt einen oder mehrere Vertreter aus der Gruppe Genistein, Daidzein oder Glycoside davon oder Metabolite oder Derivate davon umfasst.

7. Verwendung wie in Anspruch 1 oder Anspruch 2 beansprucht, wobei das Isoflavon-Phytoöstrogen Genistein und/oder Biochanin A: Daidzein und/oder Formononetin umfasst, die in einem Verhältnis von ungefähr 1:2 bis 2:1 vorhanden sind.

8. Verwendung wie in einem der Ansprüche 1 bis 7 beansprucht, wobei die Isoflavon-Phytoöstrogene in einer Menge von ungefähr 20 mg bis 200 mg pro Dosiseinheit vorhanden sind, wobei die Menge gegebenenfalls 50 mg bis 150 mg beträgt.

9. Verwendung wie in einem der vorangehenden Ansprüche beansprucht, wobei die Verabreichung des Medikaments wenigstens täglich Ober einen Zeitraum von wenigstens einem Monat erfolgt.

10. Verwendung wie in einem der vorangehenden Ansprüche beansprucht, wobei der Extrakt Coumestane, Lignane und Flavone einschließt.

11. Verwendung wie in einem der vorangehenden Ansprüche beansprucht, wobei die Dosierungseinheitsform eine Tablette oder Kapsel ist.

Revendications

1. Utilisation d'un extrait du phyto-oestrogène isoflavone de soja ou de trèfle, pour fabriquer un médicament pour administration sous forme de dosage unitaire pour le traitement du syndrome prémenstruel, des symptômes associés à la ménopause ou du cancer de la prostate.

2. Utilisation selon la revendication 1, dans laquelle le médicament comprend en outre au moins un excipient adapté sur le plan diététique.

3. Utilisation selon la revendication 1 ou 2, dans laquelle le phyto-oestrogène isoflavone est extrait du soja.

4. Utilisation selon la revendication 3, dans laquelle le phyto-oestrogène isoflavone est extrait d'hypocotyles de soja.

5. Utilisation selon la revendication 1 ou 2, dans laquelle le phyto-oestrogène isoflavone est extrait du trèfle.

6. Utilisation selon l'une quelconque des revendications 1 à 4, dans laquelle l'extrait de phyto-oestrogène isoflavone comprend un ou plusieurs parmi la génistéine, la daidzéine, ou leurs glycosides, ou leurs métabolites ou leurs dérivés.

7. Utilisation selon la revendication 1 ou la revendication 2, dans laquelle le phytooestrogène isoflavone comprend la génistéine et/ou la biochanine A:la daidzéine et/ou la formononétine, présentes selon un rapport d'environ 1:2 à 2:1.

8. Utilisation selon l'une quelconque des revendications 1 à 7, dans laquelle les phyto-oestrogènes isoflavones sont présents en une quantité d'environ 20 mg à 200 mg par dose unitaire, facultativement où la quantité est de 50 mg à 150 mg.

9. Utilisation selon l'une quelconque des revendications précédentes, dans laquelle l'administration du médicament est administrée au moins quotidiennement pendant au moins un mois.

10. Utilisation selon l'une quelconque des revendications précédentes, dans laquelle l'extrait inclut des coumestanes, des lignanes et des flavones.

11. Utilisation selon l'une quelconque des revendications précédentes, dans laquelle la forme de dosage unitaire est un comprimé ou une capsule.

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 93/23069
A61K 37/24, 35/78	A1	(43) International Publication Date: 25 November 1993 (25.11.93)

(21) International Application Number: PCT/AU93/00230

(22) International Filing Date: 19 May 1993 (19.05.93)

(30) Priority data:
PL 2511 19 May 1992 (19.05.92) AU

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(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With amended claims and statement.

(54) Title: HEALTH SUPPLEMENTS CONTAINING PHYTO-OESTROGENS, ANALOGUES OR METABOLITES THEREOF

(57) Abstract

Compositions enriched with natural phyto-oestrogens or analogues thereof selected from Genistein, Daidzein, Formononetin and Biochanin A. These may be used as food additives, tablets or capsules for promoting health in cases of cancer, pre-menstrual syndrome, menopause or hypercholesterolaemia.

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HEALTH SUPPLEMENTS CONTAINING PHYTO-OESTROGENS, ANALOGUES OR METABOLITES THEREOF

TECHNICAL FIELD

This invention relates to natural products containing phyto-oestrogens, or phyto-oestrogen metabolites, which have various beneficial physiological effects in man, and which have a variety of uses, such as to promote good health and as a dietary additive, for example.

BACKGROUND ART

The particular product in accordance with the invention is an extract of certain plants with the particular purpose of enrichment for phyto-oestrogens, both in their natural state and their closely related derivatives and metabolites.

Plants which are used as foodstuffs or medicinal herbs contain a wide variety of chemicals which are assimilated into the body following ingestion. Some of these chemicals are important nutrients for man and animals (e.g. fats, carbohydrates, proteins, vitamins, minerals) while others have none, or little or no known nutritional value. The phyto-oestrogens hitherto have fallen into this latter category of no known nutritional value.

There are 3 principal classes of phyto-oestrogens, viz. isoflavones, lignans, and coumestans. The isoflavones are thought to have a broad range of biological functions in plants, although these are poorly understood. However, two particular functions are recognised - (a) as phytoalexin or stressor chemicals which are secreted by the plant in response to attack by parasites such as insects, fungi, viruses, etc and which display activity against these parasites, and (b) chemicals which encourage colonisation of nitrogen-fixing bacteria on the roots of legumes. The biological functions in plants of the lignans and coumestans is not generally understood.

The different types of phyto-oestrogens are as follows.

Type 1 phyto-oestrogens - (isoflavones)

Isoflavones appear to be widely distributed in the plant kingdom and over 700 different isoflavones are described. However, the isoflavones which display oestrogenic activity belong to a small sub-group and are restricted almost exclusively to the *Leguminosae* family. The known oestrogenic isoflavones are daidzein, formononetin, genistein and biochanin A. In

common human foodstuffs such as soya, chickpeas, lentils and beans, the total levels of the oestrogenic isoflavones range between about 40 and 300 mg per 100 g dry weight.

In the raw plant material, isoflavones occur principally as glycosides. Following ingestion by man and animals, the glycoside moiety is hydrolysed free by a combination of gastric acid hydrolysis and fermentation by intestinal bacteria. Some of the isoflavones in the aglucone form are absorbed directly and circulate in the blood, while the remainder are metabolised by intestinal fermentation to a variety of compounds which are also absorbed. The absorbed isoflavones and their metabolites appear to undergo little or no further metabolism in the body, being readily transported in the bloodstream, and ultimately being excreted in the urine.

Type 2 phyto-oestrogens (lignans).

Lignans are widely distributed in the plant kingdom. Over one hundred lignans are described and they are reported in common human foodstuffs such as cereals, fruits and vegetables. Oilseeds such as flax (linseed) have the highest known levels at 20-60 mg/100 g dry weight, while cereals and legumes have much lower levels at 0.3-0.6 mg/100 g, and vegetables even lower levels at 0.1-0.2 mg/100 g. The most common lignan described is metairesinol. Dietary lignans also appear to be metabolised fairly efficiently within the gut by bacterial fermentation, yielding metabolites such as enterodiol and enterolactone which are absorbed into the bloodstream and excreted in the urine.

Type 3 phyto-oestrogens (coumestans).

Compared to isoflavones and lignans, oestrogenic coumestans appear to have a relatively restricted distribution in plants and generally occur at much lower levels. Alfalfa, ladino clover and some other fodder crops such as barrel medic may have significant levels and have been reported to cause reproductive dysfunction in grazing animals. In the human diet, the important sources of coumestans are sprouts of soya and alfalfa where levels up to 7 mg/100g dry weight are reported. Whole soyabeans and other common foodstuff legumes contain levels of approx. 0.12 mg/100 g dry weight and most of that is concentrated in the seed hull which commonly is removed in the preparation of human foodstuffs.

Type 4 phyto-oestrogens (oestrogens).

These are compounds closely related to animal oestrogens such as oestrone, oestradiol and

oestriol. These have been described in plants such as liquorice, apple, French bean, pomegranate and date palm. Little is known of the metabolism and biological significance of these chemicals in humans and animals.

The full range of biological effects in animals of these dietary phyto-oestrogens has received only recent study. A primary effect appears to be associated with their close structural relationship to naturally-occurring oestrogens which allows the phyto-oestrogens to mimic the effects of the endogenous oestrogens. The known biological effects of phyto-oestrogens can be summarised thus:

In vitro

(a) bind to both cytoplasmic and nuclear membrane (Type II) oestrogen receptors on human tissues;

(b) strongly compete with oestrogens for oestrogen receptors, but only weakly stimulate those receptors;

(c) strongly stimulate the production of sex hormone-binding globulin (SHBG) from human cells;

In vivo

(d) weakly oestrogenic in animals;

(e) competitively-inhibit the response of tissue to oestrogens.

The three major types of phyto-oestrogens appear to act at the cellular level in a similar manner, that is through interaction with cell surface oestrogen receptors. In the body, naturally-occurring oestrogens circulating in the blood largely exert their activity by interaction with oestrogen receptors on cell surfaces; such interactions then triggering a particular biological function of that particular cell. Phyto-oestrogens are able to bind to those oestrogen receptors because the structure of these compounds so closely resembles the endogenous oestrogens, but unlike the animal oestrogens, phyto-oestrogens only weakly activate the oestrogen receptor.

As a result of phyto-oestrogens and endogenous oestrogens competing for the oestrogen-binding sites on cells, the more weakly oestrogenic phyto-oestrogens can be considered to have an anti-oestrogenic effect. This phenomenon is known as competitive-inhibition, by which is meant that the biological effect of an active substance is impaired by the competitive

binding to a target receptor of a similar but less active compound.

Thus a primary biological effect of phyto-oestrogens is held to be competitive inhibition of endogenous oestrogens. However, another more direct effect is the stimulation of synthesis of SHBG in the liver, as occurs with orally administered synthetic steroidal oestrogens. High levels of dietary phyto-oestrogens are thought to be responsible for the higher SHBG levels seen in vegetarians and in cultures maintaining traditional (high legume-containing) diets.

At high levels, dietary phyto-oestrogens can have profound physiological effects. An example of this is sheep and cattle grazing pastures containing a high proportion of subterranean clover or red clover which can contain levels of phyto-oestrogens as high as 5% of the dry weight of the plant. As a result of the competitively-inhibitory effect of the dietary phyto-oestrogens on endogenous oestrogen function in the hypothalamus, male and female sheep and cows can develop androgenic symptoms.

Such high dietary levels of phyto-oestrogens, however, are rare. It is far more common that most animal and human diets contain low to moderate levels of phyto-oestrogens, and there is growing epidemiological evidence that such levels have a beneficial effect on human health.

In most traditional human diets in developing countries, the principal phyto-oestrogens consumed are isoflavones because of the generally high reliance on legumes (also known as pulses) as a source of protein. The general consumption rates (g/day/person) for legumes for different regions currently are approximately: Japan (50-90), India (40-80), South America (30-70), North Africa (40-50), Central/Southern Africa (20-50) and Southern Mediterranean (30-60). Legumes also are a source of lignans and, to a much lesser extent, coumestans, and the additional cereal and vegetables in the diet would also boost the lignan intake. However, the isoflavone intake in these traditional cultures with high legume consumption would typically be much in excess of either lignan or coumestan intake.

The major types of legumes used in traditional diets include soya, chickpeas, lentils, ground nuts, beans (e.g. broad, haricot, kidney, lima, navy), and grams (bengal, horse and green).

In Western, developed countries, the daily intake of dietary phyto-oestrogens generally is

negligible to low. In Western Europe, North America and Australasia, legumes were a major source of protein for the majority of the populations up to the end of the 19th century. From that time, legume consumption has declined significantly, being replaced in the diet with protein of animal origin. Average legume consumption in these regions currently is between 5-15 g/day/person with a significant proportion of the population ingesting little to no legumes or other phyto-oestrogen containing foods on a regular basis. Moreover, the types of legumes consumed in these regions (e.g. garden peas, French beans) have a typically lower isoflavone content than legumes such as soya and chick peas.

Based on typical consumption rates and types of foodstuffs consumed, the typical phyto-oestrogen intake (mg/day) for different regions can be calculated approximately as

	Isoflavones	Lignans	Coumestans
Japan	50-300	2-5	0.5
Australia	2-25	1-5	0.2

Thus it can be seen that regions which have maintained traditional diets have a higher average daily intake of phyto-oestrogens, particularly isoflavones, compared to western countries. People in communities such as Japan or developing countries with high legume intake excrete substantially higher phyto-oestrogen metabolites in their urine compared to people in Western countries. Within the latter, vegetarians also excrete higher phyto-oestrogen metabolite levels than do those consuming a more typical, omnivorous Western diet.

The presence of relatively large amounts of phyto-oestrogen metabolites in urine serves to highlight their potential biological significance. It has been shown that total urinary excretion of isoflavones and their active metabolites in people consuming moderate amounts of legumes is greatly in excess (up to 10,000 x) of steroidal oestrogen levels. So that while the oestrogenicity of isoflavones to oestrogen receptors is only about 1% that of endogenous oestrogens, this weaker effect is off-set by the much higher blood levels of the isoflavones.

It is known that legumes have formed an important part of the human diet over the past 20,000-30,000 years. It therefore follows that human metabolism has evolved over at least this period in the presence of relatively large levels of dietary phyto-oestrogens, particularly

isoflavones. Given the known biological effects of phyto-oestrogens, it also follows that endogenous oestrogen metabolism and function has evolved in the face of significant competitive inhibiting effects of phyto-oestrogens. It has been speculated that the presence of significant dietary levels of phyto-oestrogens in recent human evolution has led to a degree of adaption by tissues responsive to reproductive hormones to these dietary components. That is, both the rate of production and/or the function of endogenous oestrogens may be either dependent upon or influenced by the presence of phyto-oestrogens in the body. It follows therefore that a relative deficiency of dietary phyto-oestrogens could be expected to lead to an imbalance of endogenous oestrogen metabolism.

There is increasing interest in the likely contribution of a relative deficiency of dietary phyto-oestrogens to the development of the so-called "Western diseases", especially cancer of the breast, benign (cystic) breast disease, cancer of the uterus, cancer of the prostate, cancer of the bowel, pre-menstrual syndrome, menopausal syndrome, and atherosclerosis. All of these diseases are associated to a greater or lesser extent to oestrogen metabolism, and oestrogen function is either known or is suspected to play a role in their aetiology and/or pathogenesis.

Each of these diseases occurs at much higher incidence in Western, developed countries than it does in developing communities. Moreover, it is thought that in Western communities, the incidences of each have risen over the past century. It is also generally held, that of all the environmental factors likely to be contributing to this phenomenon, diet is the principal factor. Of those dietary components with the potential to influence the aetiology of oestrogen-related disease, there is a growing awareness that phyto-oestrogens may have important potential.

The beneficial effects of phyto-oestrogens on human health are thought to derive from at least two principal function, those being (i) competitive-inhibition of the function of endogenous oestrogens, and (ii) the stimulation of production of SHBG. SHBG plays an important role in primates in binding and transporting the reproductive hormones (oestrogens, androgens) in blood so that the availability of reproductive hormones is regulated to a large degree by SHBG levels. Higher SHBG levels are considered beneficial in leading to a reduction in both blood levels of unbound (and unregulated) reproductive hormones and metabolic clearance rates of the hormones. Although isoflavones are potent stimulators of SHBG synthesis, they only weakly bind to SHBG, so that the increased SHBG levels resulting from the dietary

isoflavones are largely available for binding to endogenous oestrogens.

In terms of directly identifying the beneficial effects of phyto-oestrogens in amelioration of any or all of the "Western diseases", there are only two examples. In one example, the diets of women, with menopausal syndrome were supplemented with foodstuffs (soya, linseed, red clover) high in phyto-oestrogens, and an alleviation of menopausal symptoms to an extent similar to that obtained with replacement therapy with synthetic oestrogens was achieved; that effect was ascribed to the phyto-oestrogen content of the supplement. In the other example, legumes such as soya and various pulses have been shown to have a hypocholesterolaemic effect in humans; this effect has not been ascribed to phyto-oestrogens, although purified isoflavones do have a hypocholesterolaemic effect in animals with artificially-induced hypercholesterolaemia.

In summary, it could reasonably be deduced that the inclusion of greater levels of foodstuffs high in phyto-oestrogens in the standard diets of men and women in developed countries could be expected to redress a general imbalance of endogenous reproductive hormone metabolism, thereby reducing the predisposition of those communities to the above diseases. While there are various types of phyto-oestrogens which may be suitable to this end, the large discrepancy in isoflavone consumption between communities with Western and traditional diets suggest that foodstuffs with high isoflavone content are of prime interest.

However it is unrealistic to expect that public education programmes would readily convert communities in developed countries from a diet where the protein content is predominantly animal-derived, to one where the protein is predominantly legume-derived. Moreover, the legumes which are commonly consumed in developed countries are relatively poor sources of phyto-oestrogens and the general acceptance in the community of less well-known legumes with higher phyto-oestrogen content would be necessarily a slow process. Also, the highly variable levels of phyto-oestrogens in foodstuffs relating to plant strain type, degree of plant maturity, and climatic and other environmental conditions suggests that the supply of an assured amount of phyto-oestrogens through the use of whole foodstuffs may be difficult.

An alternative strategy is to make available either (i) phyto-oestrogens in a purified form, or (ii) foodstuffs which are enriched for phyto-oestrogens. In this way, the phyto-oestrogen could

be added to the diet in a convenient form as a supplement without requiring any substantive change to the diet.

DISCLOSURE OF INVENTION

The present invention concerns a health supplement specifically enriched for isoflavones selected from genistein, daidzein, formononetin and biochanin A, or their natural glycoside form, or their analogues, in sufficient amounts to improve the health of a human.

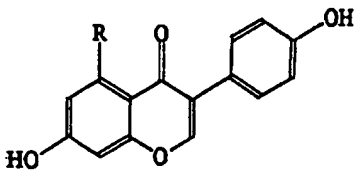
Preferably the supplement contains an excipient, a diluent, a carrier or the like, or else the supplement is mixed with food or can be consumed directly. It is also preferred that foodstuffs, are readily available, have no known toxic components, and are rich sources of isoflavones; such foodstuffs preferably being red clover or soya. It is also preferred that the ratio of genistein and/or it methylated derivative biochanin A to daidzein and/or its methylated derivative formononetin is between 1:2 to 2:1. Other plant components with oestrogenic activity including lignans, coumestans and flavones may also be present in the extract, but it is held that these are of secondary importance to the predominant isoflavones. The term phyto-oestrogens is used hereafter to indicate a predominance of isoflavones with lesser amounts of lignans, coumestans and flavones.

The invention also concerns a method of improving the health of a human by administering to the human a sufficient amount of phyto-oestrogen. Ideally, the phyto-oestrogen is administered regularly on a daily basis over a sufficient period such as at least a month. The health conditions which may be prevented or ameliorated include cancer of the breast, cancer of the prostate, cancer of the uterus, cancer of the bowel, benign (or cystic) breast disease, pre-menstrual syndrome (also known as pre-menstrual tension), or adverse symptoms associated with menopause in women. The method and supplement in accordance with the invention also improves the health of a human having elevated levels of blood cholesterol. The product also is useful in avoiding or ameliorating cancer in persons. The symptoms produced by these conditions and the general well-being is also improved by the use of these supplements.

The phyto-oestrogen in accordance with the invention may be obtained from a number of different sources. Preferably the phyto-oestrogens are extracted from a clover such as red clover or subterranean clover or from soya which contain high levels of phyto-oestrogens.

However, any source rich in phyto-oestrogens may be used instead, if desired.

Various different isoflavones have been identified from these sources - they are principally genistein, biochanin A, daidzein, formononetin and glycitein. In plants these compounds occur principally in a glycoside form bound to sugars such as glucose, with smaller amounts present as the aglucone forms. The formulae of the isoflavones are:



daidzein R = H
genistein R = OH

The structure of biochanin A is the same as for genistein but with a 4'-methoxy group, and similarly formononetin has the same structure as daidzein, but with a 4'-methoxy group.

Following ingestion by humans, the glycosidic isoflavones are hydrolysed to the aglucone form and biochanin A and formononetin are demethylated by bacterial fermentation to genistein and daidzein respectively. A small proportion of these free isoflavones are absorbed directly from the bowel and circulate in the blood. The bulk of the isoflavones, however, remain in the bowel and undergo fermentation to form various metabolites which also are absorbed into the bloodstream. The principal metabolites which have been identified are equol and O-*des*methylangolensin.

In vitro and *in vivo* studies have indicated that genistein, biochanin A, equol, daidzein, formononetin all have oestrogenic activity in descending order. O-*des*methylangolensin is only very weakly oestrogenic and glycitein is non-oestrogenic.

In animal and *in vitro* studies, genistein has been shown to have greater oestrogenic/anti-oestrogenic activity and SHBG-stimulating capacity than the other isoflavones or their metabolites (approximately 10 times that of daidzein and formononetin). However, the full range of biological effects of the different isoflavones have yet to be fully determined, and in particular their relative efficacies in the different biological effects such as oestrogenicity,

hypocholesterolaemia, anti-angiogenesis, anti-oxidation, anti-carcinogenesis for example are not yet fully known.

It is thought that because the methyl forms (biochanin A and formononetin) ultimately are largely demethylated to their principals, genistein and daidzein, with improved biological efficacy, then it is unimportant whether the isoflavones are present in the claimed product in the methylated or demethylated forms.

Given that the relative biological importance of the two isoflavone groups (being genistein and daidzein) to human health remains unclear, and that each might indeed have different importance, plus the fact that both isoflavones are present in the diet in approximately equal proportions, then it is prudent that both isoflavones be present in the claimed product in approximately equal proportions.

Any leguminous plants such as detailed here could be used as sources of phyto-oestrogens (principally isoflavones with lesser amounts of lignans and coumestans): Indian liquorice (*Abrus precatorius*); various species of *Acacia spp.* including, *A. aneura, A. cibaria, A. longifolia*, and *A. oswaldii*; ground nut (*Apio tuberosa*); ground pea (*Arachis hypogea*); milk vetch (*Astragalus edulis*); marama bean (*Bauhinia esculenta*); sword bean (*Cajanus cajan indicus*); jack bean (*Canavalia ensiformis*); sword bean (*Canavalia gladiata*); seaside sword bean (*Canavalia rosea*); various *Cassia spp.* including *C. floribunda, C. laevigata*, and *C. occidentalis*; carobbean (*Ceratonia siliqua*); chick pea (*Cicer arietinum*); yebnut (*Cordeauxia edulis*); various *Crotalaria spp.* including *C. laburnifolia*, and *C. pallida*; cluster bean (*Cyamopsis psoralioides*); tallow tree (*Detariaum senegalense*); sword bean (*Entada scandens*); balu (*Erythrina edulis*); soyabean (*Clycine max*;) inga (*Ingaedulis*); Polynesian chestnut (*Inocarpus fagifer*); hyacinth bean (*Lablab purpureus*); grass pea or Indian vetch (*Lathyrus sativus*); cyprus vetch (*Lathyrus ochrus*); lentil (*Lens culinaris*); jumping bean (*Leucaenal eucocephala*); various *Lupinus spp.* including *L. albus, L. luteus, L. angustifolium, L. mutabilis*, and *L. cosentinii*; ground bean (*Macotylma geocarpa*); horse gram (*Macrotyloma uniflorum*); alfalfa (*Medicago sativa*); velvet bean (*Mucuna pruriens*); yam beans (*Pachyrhyzuz erosus, P. tuberosus*); African locust bean (*Parkia clappertoniana*); *Parkia speciosa*; oil bean tree (*Pentaclethra macrophylla*); various *Phaseolus spp.* including *P. acutifolius, P. vulgaris, P. luntus, P. coccineus, P. adenathus, P. angulris, P. aureus, P. calcaratus, P. mungo*, and *P.*

polystachyus; garden pea (*Pisum sativum*); djenko bean (*Pithecolobium lobatum*); mesquite (various *Prosopis spp.*); goa bean (*Psophocarpus scandens, P. tetragonolobus*); various *Psoralea spp.*; *Sesbania bispinosa*; yam bean (*Sphenostylis stenocarpa*); tamarind (*Tamarindus indica*); fenugreek (*Trigonella foenum-graecum*); vetches (various *Vivia spp.* including *V. sativa, V. atropurpurea, V. ervilia,* and *V. monantha*); broad bean (*Vicia faba*); black gram (*Vigna mungo*); various *Vigna spp.* including *V. radiata, V. aconitifolia, V. adanatha; V. angularus, V. tribolata, V. umbelata,* and *V. unguiculata*; and, earth pea (*Voandzeia subterranea*).

The ideal sources of phyto-oestrogens for preparation of a supplement in accordance with the invention are preferably those which (i) are readily available, (ii) are relatively inexpensive, (iii) are readily and economically processed so as to yield the extract, (iv) have a high isoflavone content so as to provide high yields, and (v) have no known toxic components requiring selective removal or inactivation.

Certain clovers, such as red clover (*T. pratense*) and subterranean clover (*T. subterranean*) are the preferred sources. On a dry weight basis, these clovers contain the highest amounts of oestrogenic isoflavones of all legumes tested to date with levels of 3-5 g% (*T. subterranean*) and 1-3 g% (*T. pratense*). In comparison, soya flour has a level of 0.15-0.30 g%, lentils (0.08-0.12 g%), chick peas (0.07-0.13 g%), and garden peas (0.02-0.03 g%). Thus it can be seen that clovers contain approximately at least 10-30 times by weight the isoflavone content of other commonly available, human leguminous foodstuffs meaning that for manufacturing purposes, the yield of isoflavones per unit weight of plant material is many times greater from clover than from other legumes.

Red clover and subterranean clover also are common fodder crops and are readily grown and are widely available. Clovers also are comparatively cheaper ($200/tonne) than crops such as soya and lentils ($500/tonne).

With clovers, the isoflavones are recovered from the leaf rather than from the seed in the case of soya, beans, nuts and grams. This provides a substantially higher yield of isoflavones per unit area of pasture for clovers compared to other legumes because of the greater leaf matter compared to seed matter recovered per plant.

Clovers also have an extended growing season, and faster growth rates compared to those legumes such as soya, lentils or chick peas where the seed is the end-product. Clover can be cropped for its leaf content repeatedly over a single growing season. An additional benefit of this is that as phyto-alexins, the isoflavone content increases in response to the stress of cropping.

Thus it can be seen that in clovers versus other legumes provide a combination of (a) higher isoflavone content per dry weight of plant, (b) a higher yield of dry matter containing isoflavones per plant, and (c) a higher yield of dry matter per hectare.

An additional feature of clovers is that there are wide varieties of cultivars with widely differing isoflavone levels and types. This allows blending of different cultivars to achieve the desired ratio of the different isoflavones, although it is equally possible to use a single cultivar which provides the desired ratio.

Other legumes such as soyabean flour may be used for enrichment of phyto-oestrogens but the substantially poorer (approx. 10%) yield of isoflavones compared to clovers means that the manufacturing costs are substantially greater and there is substantially greater amounts of waste products which requires disposal or further treatment for re-use as a foodstuff. An alternative, however, to the use of whole soya for this purpose, is to use the hull and hypocotyl (or germ) of the whole soyabean. The hull and hypocotyl represent only a small proportion by weight.(8% and 2% respectively) of the intact bean. However, the coumestrol content of soya is concentrated in the hull, and the daidzein content of soya is concentrated in the hypocotyl. The two cotyledons which comprise the bulk of the soyabean (90% by weight) contain the bulk of the genistein content of soya. During standard processing of soyabeans, the hulls being a fibrous component with little or no perceived nutritional value normally are separated and removed by physical means. The hypocotyls become separated following the splitting of the cotyledons, and while these currently generally are not deliberately isolated, they may be separated and isolated by passing the disturbed soyabeans over a sieve of sufficient pore size to selectively remove the small hypocotyl. The hypocotyl contains approx. 1.0-1.5 g% isoflavones (95% daidzein, 5% genistein). The raw hypocotyl and hull material can be ground or milled to produce, for example, a dry powder or flour which then could be either blended or used separately as a dietary supplement in a variety of

ways including, for example, as a powder, in a liquid form, in a granulated form, in a tablet or encapsulated form, or added to other prepared foodstuffs. Alternatively, it could be further processed to yield an enriched extract of phyto-oestrogens. Either or both of these materials also could be added to other leguminous material such as clover to provide the invention.

In plants, the oestrogenic isoflavones are restricted principally to the leaf, fruit and root; the stem and petiole contain very little. With soya and other common human legume foodstuff crops, the leaves are rarely regarded as foodstuff; indeed with these crops, the plants normally are allowed to die and dry out before the seed crop is harvested. Nevertheless, the fresh leaves of these crops could be regarded as a source of phyto-oestrogens for the invention although the much lower isoflavone content of the leaves of these crops compared to clovers, plus their generally slow growth compared to clovers, suggests that they would not be a preferred source of large-scale isoflavone enrichment.

To provide a similar amount of isoflavone to that contained in most traditional legume-rich diets (50-100 mg oestrogenic isoflavones/day) would require an average daily consumption of 3-6 g dry weight or 15-30 g wet weight of specially selected cultivars of clover with particularly high isoflavone levels. Clover grasses generally are not eaten by humans, except to a limited extent as sprouts of some of the pleasanter tasting varieties. Isoflavones are intensely astringent and are responsible in large part for the bitter taste of legumes. Thus the types of bean sprouts, clover sprouts and alfalfa sprouts generally available have been selected on the basis of cultivar and of age for pleasant taste, and in so doing inadvertently have been selected for low isoflavone content. Of the sprouts currently available in Western countries for human consumption, between approx. 100-250 g would need to be consumed daily to provide a dosage of 50-100 mg isoflavones. Certainly clovers and other legume sprouts are not generally eaten in such sufficient quantities by humans to obtain the advantages of the present invention.

The invention also concerns formulations containing the phyto-oestrogens discussed above together with a dietary suitable excipient, diluent, carrier, or with a food. Ideally the formulation is in the form of a pill, tablet, capsule, or similar dosage form.

The formulations may be a variety of kinds, such as nutritional supplements, pharmaceutical

preparations, vitamin supplements, food additives or foods supplemented with the specified active phyto-oestrogens of the invention, liquid or solid preparations, including drinks, sterile injectable solutions, tablets, coated tablets, capsules, powders, drops, suspensions, or syrups, ointments, lotions, creams, pastes, gels, or the like. The formulations may be in convenient dosage forms, and may also include other active ingredients, and/or may contain conventional excipients, carriers and diluents. The inclusion of the subject phyto-oestrogens in herbal remedies and treatments is also a preferred part of the invention.

The invention is also directed to the amelioration, prevention, or of various conditions responsive to treatment with the phyto-oestrogen substances of the invention. The preferred amounts to be administered to the human fall within 20 - 200 mg on a daily basis. More preferably the dosage is from 50 - 150 mg on a daily basis, and most preferably at a dosage of about 100 mg. If desired greater dosages can be administered for therapeutic reasons. In contrast to prior practices such high dosages were not possible. For example, dosages of up to or greater than 1000 mg may be suitable to treat some conditions. In order to obtain the benefits of the invention, the treatment with the isoflavones should continue for a considerable period, ideally for at least a month, and ideally continuously for the whole period for which the health improvement advantages should accrue.

The product according to the present invention yields a constant and accurately known amount of isoflavones. The product is also ideally a natural product, which has advantages for consumer acceptance, and in accordance with the supposed theory behind the invention may very possibly be one of the main causes for its beneficial effects. Whole legumes have a widely variable isoflavone content due to two main causes: the type of legume and the environmental effect. The type of legume typically has a wide range of isoflavone content. The miligram of isoflavone per hundred gram of whole foodstuff (dry weight) is given in the following table:

Soya Products	
- Whole Soya	150 - 300
- Soya Milk	25 - 40 (mg per 200 ml)
- Tofu	55 - 95
Lentils	80 - 120
Chickpeas	70 - 130
Broad beans	15 - 20
Garden peas	15 - 25

Thus common leguminous foodstuffs consumed in Western countries (broad beans, garden peas etc) have relatively low oestrogenic isoflavone content and exceptionally large amounts of these would need to be consumed daily to approximate those isoflavone levels consumed in traditional diets. Most Western cultures do not traditionally eat legumes with high isoflavone contents, and those soya products (milk, tofu etc) which are becoming increasingly popular in Western countries, also have relatively low isoflavone levels compared to whole soya, indicating that relatively large amounts of these would need to be consumed on a regular basis to deliver the required isoflavone levels.

The enviromental effect arises because the isoflavone levels in any species of plant depend greatly on the age of the plant, the climatic conditions where it is grown, the fertiliser and so forth. Therefore constant and consistant dosage is very difficult with ordinary whole foodstuffs. The accurately determined quality and quantity of the active isoflavones in the product, and its easy consumability when compared with the almost impossible task of eating huge amounts of often practically inedible foods, is therefore an import feature of the invention for preventing and helping in overcoming various health problems.

Among the various health problems, the treatment or prevention of high blood cholesterol levels, and the treatment of PMS and menopausal symptoms is especially important.
The product of the invention modulates the production and/or function of endogenous sex hormones in humans to modify or produce health improving effects, including the following: (i) lowered levels of various blood lipoproteins including, for instance, low-density and very-low-density cholesterol leading to reduced risk of development of atherosclerosis; (ii) reduced risk of development of cancer of the prostate; (iii) reduced risk of cancer of the

breast; (iv) reduced risk of development of cancer of the uterus; (v) reduced risk of development of cancer of the large bowel; (vi) reduced risk of development of the syndrome in women commonly referred to pre-menstrual syndrome (PMS), which includes pre-menstrual tension (PMT); (vii) reduced risk of development of many untoward symptoms (including dry vagina, peripheral flushing, depression etc) commonly associated in women with menopause; and for treating benign breast disease in women (benign or cystic breast disease associated with non-malignant swelling and tenderness of breast tissue). The invention therefore is directed to a method for the prophylaxis or treatment of a human, to combat conditions associated with phyto-oestrogen deficiency, which comprises administering to the human an effective amount of phyto-oestrogen principally isoflavone but which might also include relatively smaller amounts of lignans and coumestans, ideally in a concentrated form, wherein the isoflavones include genistein, and/or biochanin A, and/or daidzein, and/or formononetin.

Cancer of the breast generally is considered to be associated with oestrogenic dysfunction. Breast cancer cells may display more oestrogen receptors than normal breast cells and stimulation of these receptors by endogenous oestrogens is thought to be a prime source of stimulation of their malignant growth. Currently synthetic anti-oestrogens are being used to prevent or treat the growth of malignant breast cells. Isoflavones are potent anti-oestrogens that could be expected to help prevent or to successfully treat breast cancer. It has been reported that the risk of breast cancer in western societies is indirectly proportional to the level of phyto-oestrogens in the diet and to the amounts of phyto-oestrogen metabolites excreted in the urine.

Cancer of the prostate generally is considered to be associated with sex hormone dysfunction and the growth of prostatic cancer cells is influenced by oestrogens and androgens. The incidence of prostatic cancer is low in communities with high legume intake and, conversely, is high in Western socieites. Phyto-oestrogens are though to protect from development of prostatic cancer. One mechanism may be the effect of phyto-oestrogens on lowering the proportion of unbound:bound reproductive hormones in the blood. However, there is other evidence to suggest that phyti-oestrogens, particularly isoflavones, can have a direct influence on certain cellular enzymes within prostatic cells.

Pre-menstrual syndrome has uncertain aetiology and pathogenesis, although most certainly is

associated with reproductive hormone dysfunction. It also is a syndrome which has reportedly lower incidence in communities maintaining traditional high-legume diets. It is proposed that phyto-oestrogens will alleviate this condition by restoring balance to oestrogen metabolism.

Menopausal syndrome is associated with changes in the oestrogen profile in the body with advancing age. Adverse clinical symptoms may be treated with oestrogen replacement therapy. There is evidence that foodstuffs high in phyto-oestrogens are a suitable alternative to synthetic hormones in this respect, producing alleviation of adverse clinical symptoms. Again, it is proposed that phyto-oestrogens will function by restoring balance to oestrogen metabolism.

Benign (or cystic) breast disease has unknown aetiology. However, its association in women with certain stages of the menstrual cycle is strongly suggestive of oestrogen dysfunction. There currently is no successful treatment of this condition. Phyto-oestrogens are proposed to successfully treat this condition by restoring balance to oestrogen metabolism.
Atherosclerosis is associated with cholesterol metabolism which in turn is associated closely with oestrogen metabolism. The generally higher incidence of atherosclerosis in young men versus young women, the rising incidence in women following menopause, and the lower incidence in post-menopausal women receiving oestrogen replacement therapy, all point to the moderating influence of oestrogens on cholesterol metabolism. A prime effect of oestrogens on cholesterol metabolism is stimulation of the liver to process cholesterol, particularly the highly atherogenic low-density lipoproteins and very low-density lipoproteins, into bile salts. It is proposed that phyto-oestrogens have an important hypocholesterolaemic effect in humans. There may be a variety of mechanisms involved, but one may be the stimulation by phyto-oestrogens of cholesterol catabolism by the liver.

MODES FOR CARRYING OUT THE INVENTION

The invention is now described with reference to various examples.

EXAMPLE 1 - Preparation of Red Clover Product

Tablets were prepared using red clover in accordance with the following procedure. The raw plant material is harvested and dried; such drying being either sun-drying or from applied heat.

The dried product is then preferably chaffed, before the following extraction step, although this can be omitted if desired.

The dried material is extracted in an aqueous: organic solvent mix. The aqueous phase is required to extract the water-soluble glycoside form of isoflavones, while the organic solvent is required to solubilise the water-insoluble aglycone form. The organic solvent can be either alcohol, chloroform, acetone or ethyl acetate. The ratio of solvent in the water can be between 0.1% and 99.9%. The preferred method is to use 60% alcohol in water.

The isoflavones are extracted by exposing the plant material to the water:solvent mix. The exposure time in general terms is indirectly proportional to the temperature of the mixture. The temperature of the mix can range between ambient temperature and boiling temperature. The exposure time can be between 1 hour and 4 weeks or even longer. It has been determined that the adequate times for maximal recovery of isoflavones are 2 weeks at 50°C and 24 hours at 90°C. The supernatant is separated from the undissolved plant material and the organic solvent removed by distillation. The aqueous supernatant then is concentrated, typically by distillation.

Additional processing steps can be used, if desired, to convert the extracted natural product to capsule, tablet, or other convenient form for ingestion, using normal techniques for doing this. Otherwise the product can be packaged as a convenient food additive.

EXAMPLE 2 - preparation of soya hypocotyl product.

Soyabeans were heated in dry air so that the hull became brittle. The beans then were processed through a tumble mill which removed the hull and split the bean the two cotyledons and the small-sized hypocotyl which separated from each other. The light-weight hulls then were removed by an air stream. The small-sized hypocotyls were separated from the larger cotyledons by sieving through a steel wire mesh with apertures of 1 mm x 1 mm. This yielded approximately 87% purity of hypocotyls with 13% contamination by small cotyledon chips.

Normal soybean processing steps isolate the hulls and then these are discarded or processed

separately for use in human and animal foodstuffs. The hypocotyls normally are not separated and are processed along with the cotyledons. However, a small number of soybean processors are separating hypocotyls by the above methods in order to reduce the astringent taste of soyflour for human consumption, and currently these hypocotyls are either discarded or processed to flour for use in animal feed.

EXAMPLE 3 - effect of administering red clover extract to humans

Seven normal individuals were studied for the comparative effects of red clover extract and whole legumes on blood cholesterol levels. All the individuals were consuming a standard Western diet with minimal levels of legumes.

Three men consumed between 100-150 g haricot or navy beans daily for 3 weeks as a supplement to their normal diet. This yielded an approximate daily isoflavone dosage or between 60-100 mg.

Four other individuals (3 men, 1 woman) consumed 5 g of red clover extract containing 100 mg isoflavones daily for 3 weeks.

Total serum cholesterol levels were determined immediately before and immediately following the challenge.

	Pre-treatment	Post-treatment	% change
Beans only			
Patient 1	5.77	5.46	- 5.4
Patient 2	6.24	6.12	- 1.9
Patient 3	7.45	8.51	+14.3
Red clover extract			
Patient 5	6.53	5.90	- 9.6
Patient 6	7.43	6.63	-10.8
Patient 7	6.33	5.50	-13.1
Patient 8	6.98	7.28	+ 4.3

The red clover extract had a significantly ($P < 0.05$) greater hypocholesterolaemic effect than

did the whole beans.

Neither of the treatments produced any untoward side effects, although the whole bean eaters reported greater difficulty with compliance of treatment than did those taking the red clover extract.

EXAMPLE 4 - effect of administering soy hypocotyls to humans

Fifteen volunteers (8 women, 7 men) were given 5 g of soy hypocotyl containing (45 mg daidzein and 5 mg genistein) daily for 2 months. The hypocotyl was consumed as a powder added to the diet.

The effects on cholesterol levels are shown in the following table. The individuals are grouped according to their pre-treatment cholesterol levels (high, medium, low).

		Range (mean) unmol/L	
	n	Pre-treatment	Post-treatment
Group 1	6	6.3 - 8.4 (7.1)	5.4 - 6.5 (6.1)
Group 2	6	5.0 - 6.2 (5.5)	4.7 - 5.9 (5.1)
Group 3	3	3.3 - 4.7 (4.2)	3.4 - 4.6 (4.1)

The results show a significant fall in total cholesterol levels in those individuals with cholesterol levels considered to be at the upper end of the normal range.

In addition, 1 woman reported substantial amelioration of her benign breast disease problem associated with mid-cycle swelling and tenderness, and another woman reported regularisation of her menstrual cycle and reduced menstrual bleeding. Both of these effects were regarded as beneficial.

No other side-effects were reported as a result of the treatment.

THE CLAIMS:

1. A health supplement comprising a health supplementary amount of a phyto-oestrogen selected from genistein, daidzein, biochanin A, and/or formononetin.

2. The supplement according to claim 1 which also comprises at least one dietary suitable excipient, diluent, carrier or food.

3. The supplement according to claim 1 wherein said phyto-oestrogen is extracted from red clover.

4. The supplement according to claim 1 wherein said phyto-oestrogen is extracted from soya.

5. The supplement according to claim 4 wherein said phyto-oestrogen is extracted from soya hypocotyls.

6. The supplement according to claim 1 wherein said phyto-oestrogen comprises genistein, and/or biochanin A : daidzein and/or formononetin, present in a ratio of from about 1:2 to 2:1.

7. The supplement according to claim 1 in unit dosage form, wherein said phyto-oestrogen is present in an amount of from about 20 mg to 200 mg per dosage unit.

8. The supplement according to claim 7, where the amount is 50 to 150 mg.

9. The supplement according to any one of claims 1 to 8, which is in the form of a tablet or capsule.

10. A method of improving the health of a human which comprises administering to said human a health supplementing amount of a phyto-oestrogen selected from genistein, daidzein, biochanin A, and/or formononetin.

11. The method according to claim 10, wherein said phyto-oestrogen is extracted from red clover.

12. The method according to claim 10, wherein said phyto-oestrogen is extracted from soy.

13. The method according to claim 12, wherein said phyto-oestrogen is extracted from soy hypocotyls.

14. The method according to claim 10, whereby said phyto-oestrogen comprises genistein and/or biochanin A : daidzein and/or formononetin, present in ratio of from about 1:2 to 2:1.

15. The method according to claim 10, wherein the phyto-oestrogen is administered in an amount of from about 20 mg to 200 mg per day.

16. The method according to claim 10, wherein the phyto-oestrogen is administered in an amount of from about 50 mg to 150 mg per day.

17. The method according to claim 10 whereby the phyto-oestrogen is administered at least daily, over a period of at least a month.

18. The method according to any one of claims 10 to 17 for improving the health of a human female who has, or may develop, a condition selected from any one or more of: breast cancer, benign breast disease, pre-menstrual syndrome, or symptoms associated with menopause.

19. The method according to any one of claims 10 to 17 for improving the health of a human who has, or may develop, elevated levels of cholesterol in the blood stream.

20. The method according to any one of claims 10 to 17 for improving the health of a human who has or may develop cancer.

AMENDED CLAIMS

[received by the International Bureau on 8 October 1993 (08.10.93); original claims 1-10 amended; other claims unchanged (1 page)]

1. A health supplement comprising a health supplementary amount of a naturally occurring phyto-oestrogen selected from any two or more of genistein, daidzein, biochanin A, formononetin, and/or their glycosides.

2. The supplement according to claim 1 which also comprises at least one dietary suitable excipient, diluent, carrier or food.

3. The supplement according to claim 1 wherein said phyto-oestrogen is extracted from red clover.

4. The supplement according to claim 1 wherein said phyto-oestrogen is extracted from soya.

5. The supplement according to claim 4 wherein said phyto-oestrogen is extracted from soya hypocotyls.

6. The supplement according to claim 1 wherein said phyto-oestrogen comprises genistein, and/or biochanin A : daidzein and/or formononetin, present in a ratio of from about 1:2 to 2:1.

7. The supplement according to claim 1 in unit dosage form, wherein said phyto-oestrogen is present in an amount of from about 20 mg to 200 mg per dosage unit.

8. The supplement according to claim 7, where the amount is 50 to 150 mg.

9. The supplement according to any one of claims 1 to 8, which is in the form of a tablet or capsule.

10. A method of improving the health of a human which comprises administering to said human a health supplementing amount of a naturally occurring phyto-oestrogen selected from any two or more of genistein, daidzein, biochanin A, formononetin, and/or their glycosides.

STATEMENT UNDER ARTICLE 19

The independent claims have been restricted to define a supplement containing at least two of the naturally occurring phyto-oestrogens, including their glycosidic forms.

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PCT/AU 93/00230

Patent Office
Canberra

I, RONALD MAXWELL MAY, ASSISTANT DIRECTOR PATENT ADMINISTRATION, hereby certify that the annexed is a true copy of the Provisional specification as filed on 19 May 1992 in connection with Application No. PL 2511 for a patent by GRAHAM EDMUND KELLY filed on 19 May 1992.

I further certify that the annexed documents are not, as yet, open to public inspection.

WITNESS my hand this Twenty-Eighth day of May 1993.

RONALD MAXWELL MAY
DELEGATE OF COMMISSIONER OF PATENTS

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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

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Invention Title: Natural health supplement.

The invention is described in the following statement:

NATURAL HEALTH SUPPLEMENT

This invention relates to natural products containing phyto-oestrogens, or phyto-oestrogen metabolites, which have various beneficial physiological effects in man and other animals, and which have a variety of uses, such as to promote good health and as a dietary additive, for example.

The particular product in accordance with the invention is composed of various nutrients and chemicals, some that are important (e.g. protein, fat, carbohydrate, vitamins, minerals) and others that have little or no known nutritional value to animals and man. However, the important component in relation to the present invention are the phyto-oestrogens, or their closely related derivatives and metabolites. Phyto-oestrogens are hormones which play important roles in plant reproduction, mediating such activities as flowering and budding. Because of their functional importance, it is reasonable to expect that they may be present in all plants, although at present their presence has been sought in a limited number of plant types only, and they have been found to be present in variable but detectable levels in those plants.

There are different types of phyto-oestrogens as follows:

Type 1 phyto-oestrogens - these are compounds closely related to animal oestrogens such as oestrone, oestradiol and oestriol. They have been described in plants such as liquorice (Glycyrrhiza glabrata), apple (Malus sylvestris), French bean (Phaseolus vulgaris), pomegranate (Punica granatum), and date palm (Phoenix dactylifera).

Type 2 phyto-oestrogens - coumestans. A large number of coumestans have been isolated, but only a small number show oestrogenic activity in animals. The important ones occur in alfalfa (Medicago sativa), ladino clover (Trifolium spp.) and some other fodder crops such as barrel medic

(Medicago litteralis) where they have important effects on the reproductive performance of grazing animals. In human diet, the important sources of coumestans are sprouts of soya (Soja max) and alfalfa (Medicago sativa) where levels up to 7 mg/100 gm dry weight are seen. Whole soyabeans contain levels of approx. 120 mg/100 gm dry weight and most of that is concentrated in the seed hull.

Type 3 phyto-oestrogens - resorcylic lactones. These are compounds which are not intrinsic components of plants but are metabolites of fungal infections (particularly Fusarium spp.) associated with plants. They principally affect fodder crops where they may be significant in the health of grazing animals. Resorcylic lactones have been isolated in such foodstuffs as oats (Avena sativum), barley (Hordeum volgare), corn (Zea mays) and rice (Oryza sativa) which feature in human diets.

Type 4 phyto-oestrogens - isoflavones. These have only been isolated in significant quantities from leguminous plants such as soya (Soya max), chick pea (Cicer arietinum), and clovers (Trifolium spp.).

Phyto-oestrogens of Types 1 and 3 above generally occur at comparatively low levels in most human diets and are considered to have little, if any, significance in terms of human and animal nutrition. In contrast, coumestans, and to a greater extent, isoflavones, are far more prominent in the human diet and for that reason are thought to have some significance to human nutrition.

It is known that dietary phyto-oestrogens are absorbed by animals (including man) from the gut, then circulate in blood, and are excreted in the urine and bile. A proportion of the phyto-oestrogens are absorbed in a relatively unchanged form, while a further proportion undergo bacterial fermentation in the gut to produce a range of metabolites which are then absorbed.

Phyto-oestrogens have been shown to have a range of biological effects in animals in vivo or on animal tissues in vitro. Those effects include anti-carcinogenicity, and

anti-fungal and anti-bacterial activities. However, the potentially most significant biological effect of in animals is related to the oestrogenicity of some of the phyto-oestrogens and some of their metabolites. Some phyto-oestrogens, in particular coumestans and isoflavones, closely resemble structurally animal oestrogens and are able to mimic the activities of those animal oestrogens when injected into animals. However, while they do display oestrogenicity in animals, that effect is substantially weaker than that of the animal oestrogens. But experimental evidence indicates that the more significant biological effect of these compounds is their ability to compete with natural animal oestrogens for the oestrogen receptors on the surface of cells.

In the body, naturally-occurring oestrogens circulating in the blood exert their activity by interaction with oestrogen receptors on cell surfaces; such interaction then triggering a particular biological function of that particular cell. However, phyto-oestrogens also are able to bind to those oestrogen receptors because the structure of these compound so closely resembles the animal's own oestrogens, but unlike the animal oestrogens, phyto-oestrogens only weakly activate the oestrogen receptor.

It is likely that natural oestrogens and phyto-oestrogens compete equally for those binding sites, so in this way, the phyto-oestrogens are inhibiting the effect of oestrogens and can be considered to have an anti-oestrogenic effect. This phenomenon is known as competitive-inhibition, by which is meant that the biological effect of an active substance is impaired by the competitive binding to a target receptor of a similar but inactive compound.

At high levels, dietary phyto-oestrogens can have a profound physiological effect in animals causing severe reproductive dysfunction. An example of this is sheep grazing pastures containing certain strains of subterranean clover which can contain levels of phyto-oestrogens as high

as 5% of the dry weight of the plant. As a result of the competitive-inhibitory effect of these phyto-oestrogens on oestrogen function in the hypothalamus, both male and female sheep develop adrogenic symptoms.

Such high dietary levels of phyto-oestrogens, however, are rare. It is far more common that many animal and human diets contain just moderate levels of phyto-oestrogens and there is some evidence to support the view that moderate levels have a beneficial effect on health. In the human diet, dietary phyto-oestrogens come predominantly from legumes.

Legumes are by definition those plants that have the capability of fixing atmospheric nitrogen and for that reason yield seeds and fruit with high protein content. Coincidentally, they also appear to have relatively higher levels of phyto-oestrogens (particularly isoflavones) than do other plant types. Soya is a particularly rich source of phyto-oestrogens, containing approximately 300-1500 mg of isoflavones per 100 gm of whole soya, with the content varying according to factors such as strain variety and seasonal and growing conditions. Other common dietary legumes such as lentils, chickpeas and beans generally contain lower levels than those in soya, but still have much higher levels than that found in non-leguminous plants.

Legumes (also known as pulses) play an important dietary role in many traditional human cultures. In those cultures, the legume serves as a cheap and relatively abundant source of protein and fats. Hence, soya is a staple in Asia, beans (broad, haricot, kidney, lima, navy etc) are staples in South America, and chickpeas and lentils are staples in India and Mediterranean countries. In more affluent countries, whereas legumes (dried nuts, peas and beans) were a major source of protein up to the end of the 19th century, legume consumption has over the last 100 years declined significantly, having been replaced in the diet with protein of animal origin.

The current general consumption rates (gm/day/person) for legumes for different regions are currently approximately: Japan (90), India (50), South America (20-60), North Africa (50), Central/Southern Africa (20-50), Northern and Southern Mediterranean (20-30), Western Europe/North America/Australasia (5-15).

Thus people belonging to cultures in which legumes play an important dietary role are consuming on a daily basis significant quantities of phyto-oestrogens. Conversely, most Western developed countries currently generally have negligible to low dietary phyto-oestrogen levels because of the low level of dietary legumes, although this is a relatively recent phenomenon since up to the end of the 19th century legumes continued to be an important, albeit declining, part of the diet for the majority of the populations in Europe and North America.

It is thought that legumes have played an important part of the human diet over the past 20,000-30,000 years. It therefore follows that human metabolism have probably evolved and developed over this time in the presence of relatively large levels of dietary phyto-oestrogens, particularly isoflavones. Given the known biological effects of phyto-oestrogens, it also follows that endogenous oestrogen metabolism and function has evolved in the face of a significant competitive-inhibiting effect by phyto-oestrogens. Clinical evidence now indicates that a relative deficiency of dietary phyto-oestrogens leads to an imbalance of normal oestrogen metabolism and function in animals, including man.

It is possible that the inclusion of phyto-oestrogens such as coumestans and isoflavones in human diet during recent human evolution has resulted in some sort of adaptation of the oestrogenic metabolism in humans to the presence of these compounds. That is, both the rate of production and the function of oestrogens may be either dependent upon or influenced by the presence of phyto-oestrogens in the body. A relative deficiency of

dietary phyto-oestrogens may therefore be expected to lead to an imbalance in the levels of different oestrogens and/or the effects of those oestrogens in the body.

It is also possible that the relatively higher levels of phyto-oestrogens in the diets of traditional cultures in such developing regions as South-East Asia, India, South America and the Mediterranean which incorporate larger amounts of legumes compared to the standard diets in most developed countries, may in part account for the traditionally lower incidence in developing countries of diseases such as cancer of the breast, cancer of the bowel, cancer of the uterus, cancer of the prostate, athero-sclerosis, pre-menstrual syndrome, and menopausal syndrome, all of which are related to oestrogen metabolism to varying degrees.

To that end, the inclusion of greater levels of legumes (particularly those rich in phyto-oestrogens such as soya, chickpeas and lentils) in the standard diets of developed countries could be expected to redress a general oestrogenic imbalance in both men and women in developed countries thereby reducing the predisposition of those communities to the above diseases.

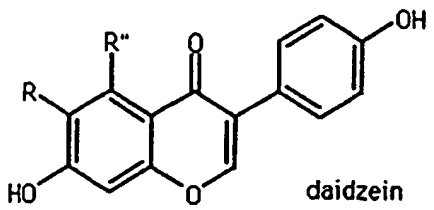
However, it is unrealistic to expect that public education programmes would readily convert communities in developed countries from a diet where the protein content is predominantly animal-derived, to one where the protein is predominantly legume-derived. An alternative strategy is to make available either (i) phyto-oestrogens in a purified form or (ii) foodstuffs which are enriched for phyto-oestrogens. In this way, the phyto-oestrogens could be added to the diet in a convenient form as a supplement without requiring any substantive change to the diet.

In soya, the coumestan content is contained principally in the hull and hypocotyl of the whole soyabean; the two cotyledons which represent the bulk (90% by weight) of the soyabean contain only minimal levels of coumestans. Similarly, isoflavones occur principally in the hypocotyl,

with the cotyledons containing much lower amounts. As the hull and hypocotyl represent just a small proportion by weight (8% and 2% respectively) of the entire soyabean, it can be seen that the phyto-oestrogens are concentrated in these two structures of the bean.

The hull and hypocotyl can be removed from whole soya beans by physical means, allowing them to separate from the cotyledons. The hulls and the hypocotyls can subsequently be separated from each other by physical means based on the higher density and smaller size of the hypocotyls.

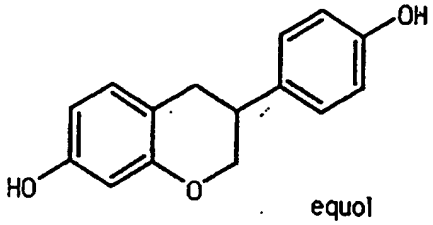
There is a further advantage in using the hypocotyl as a source of isoflavones compared to the whole soya bean, because of the relative incidences of the different isoflavones in the hypocotyl compared with the cotyledon. The isoflavone content in soy ranges from about 0.047% to 0.36% by weight. Three principal isoflavones have been identified - they are glycitein, genistein and daidzein. In plants these compounds occur principally as glycosides (glycitin, genistin, daidzin) with a smaller proportion present as aglucones (glycitein, genistein, daidzein).



daidzein R = H, R" = H
genistein R = H, R" = OH
glycitein R = OCH_3, R" = H

Following ingestion by animals, the isoflavones are hydrolysed to the free form. A small amount of these then are absorbed directly from the bowel and circulate in the blood. The bulk of the isoflavones remaining in the bowel, however, undergo fermentation by bowel bacteria to form various metabolites. These metabolites then are absorbed into the blood stream. Of the different isoflavones,

glycitein and its metabolites have negligible oestrogenic activity; genistein is oestrogenic, but its principal metabolites (para-ethylphenol and O-desmethylangolensin) have negligible oestrogenic activity; daidzein is oestrogenic, and it has two principal metabolites - O-desmethylangolensin and equol. Equol is strongly oestrogenic, being some 100 times more potent in this respect than the parent daidzein compound, or than genistein.



equol

It is therefore likely that equol is the principal source of the oestrogenic and anti-oestrogenic activity of isoflavones. In soya beans, the genistein is located predominantly in the cotyledons, while daidzein occurs predominantly in the hypocotyl.

The opportunity then exists to formulate a food supplement based on soya hulls only (as an enriched source of coumestans), or soya hypocotyls only (as an enriched source of isoflavones and moderate levels of coumestans), or a mixture of soya hulls and soya hypocotyls (as an enriched source of both coumestans and isoflavones). Similarly, these materials may be used as a source of coumestans and isoflavones for further chemical or physical extraction or purification of those compounds leading to the further enrichment or eventual purification of these compounds for the purposes listed above.

However, a preferred embodiment of the present invention is a blend of soya hulls and soya hypocotyls (in which the soya hull content of the final blend varies between 0.1% and 99.9%) without further steps to enrich the phyto-oestrogens. Such a product offers the further advantage of being a rich source of dietary soluble fibre

which is generally considered to have a beneficial effect to human health.

Soya hypocotyls compared to whole soya beans or any other whole form of legume, offer distinct advantages (i) as an enriched source of isoflavone (1.5% vs 0.3% w/w), and (ii) as a source of a proportionately greater amount of the more desirable isoflavone, daidzein.

While soya is the preferred source of phyto-oestrogens, other sources can be utilized in the invention. Soya is a preferred source of coumestan and isoflavone because of (a) the ready availability of large quantities of this material, (b) the high proportion of daidzein relative to genistein in the hypocotyl, (c) the relative ease in the separation and collection of hulls and hypocotyls, and (d) the high levels of both coumestans and isoflavones.

An alternative source of these isoflavones is subterranean clover (Trifolium spp.), many varieties of which have isoflavone levels of the order of 5% of dry weight. Compared to soya, however subterranean clovers are less advantageous because of the relative absence of coumestans.

However, other leguminous plants as detailed here could be used as sources of isoflavones and coumestans: Indian liquorice (Abrus precatorius); various species of Acacia spp. including A. aneura, A. cibaria, A. longifolia, and A. oswaldii; ground nut (Apio tuberosa); ground pea (Arachis hypogea); milk vetch (Astragalus edulis); marama bean (Bauhinia esculenta); sword bean (Cajanus cajan indicus); jack bean (Canavalia ensiformis); sword bean (Canavalia gladiata); seaside sword bean (Canavalia rosea); various Cassia spp. including C. floribunda, C. laevigata, and C. occidentalis; carobbean (Ceratonia siliqua); chick pea (Cicer arietinum); yebnut (Cordeauxia edulis); various Crotalaria spp. including C. laburnifolia, and C. pallida; cluster bean (Cyamopsis psoralioides); tallow tree (Detariaum senegalense); sword bean (Entada scandens); balu (Erythrina edulis); soyabean (Clycine max;) inga (Inga

edulis); Polynesian chestnut (Inocarpus fagifer); hyacinth bean (Lablab purpureus); grass pea or Indian vetch (Lathyrus sativus); cyprus vetch (Lathyrus ochrus); lentil (Lens culinaris); jumping bean (Leucaenal eucocephala); various Lupinus spp. including L. albus, L. luteus, L. angustifolium, L. mutabilis, and L. cosentinii; ground bean (Macotylma geocarpa); horse gram (Macrotyloma uniflorum); alfalfa (Medicago sativa); velvet bean (Mucuna pruriens); yam beans (Pachyrhyzuz erosus, P. tuberosus); African locust bean (Parkia clappertoniana); Parkia speciosa; oil bean tree (Pentaclethra macrophylla); various Phaseolus spp. including P. acutifolius, P. vulgaris, P. luntus, P. coccineus, P. adenathus, P. angulris, P. aureus, P. calcaratus, P. mungo, and P. polystachyus; garden pea (Pisum sativum); djenko bean (Pithecolobium lobatum); mesquite (various Prosopis spp.); goa bean (Psophocarpus scandens, P. tetragonolobus); various Psoralea spp.; Sesbania bispinosa; yam bean (Sphenostylis stenocarpa); tamarind (Tamarindus indica); fenugreek (Trigonella foenum-graecum); vetches (various Vivia spp. including V. sativa, V. atropurpurea, V. ervilia, and V. monantha); broad bean (Vicia faba); black gram (Vigna mungo); various Vigna spp. including V. radiata, V. aconitifolia, V. adanatha, V. angularus, V. tribolata, V. umbelata, and V. unguiculata; and, earth pea (Voandzeia subterranea).

Soya hypocotyls may be used as an enriched source of isoflavones and coumestans (0.5-1.5% dry weight) with or without the presence of additional soya hulls. Alternatively, other sources can be utilized, as listed above. This can be consumed either in the raw form (eg, natural hypocotyls), or the hypocotyls (with or without soya hulls) can be ground or milled to a powder or flour which can be used as a dietary supplement in a variety of ways including, for example, as a powder, in a liquid form, in tablet form, or added to other prepared foodstuffs. Herbal and similar types of health food and dietary supplements that include these phyto-oestrogens can also be prepared.

Equol, which is metabolized from daidzein, itself seems to be a dietary substance essential to the proper functioning of the human body, or in other words, equol (or daidzein) may be a new natural "vitamin".

The invention also includes the possibility of processing further the soy or other produce containing concentrated isoflavones and daidzein to further concentrate, or even isolate the daidzein, and if desired, to convert it into equol, for use as a new vitamin. Equol is also capable of being synthesised.

The concentrated or purified daidzein, or concentrated or pure equol can be compounded as a vitamin, in tablet, capsule, liquid or similar form, or incorporated in food as an additive, for example.

The product of the invention modulates the production and/or function of endogenous sex hormones in humans and other animals to modify or produce the following effects: (i) lowered levels of blood lipoproteins including low-density and very-low-density cholesterol leading to reduced risk of development of atherosclerosis; (ii) reduced risk of development of cancer of the prostate; (iii) reduced risk of cancer of the breast; (iv) reduced risk of development of cancer of the uterus; (v) reduced risk of cancer of development of cancer of the large bowel; (vi) reduced risk of development of the syndrome in women commonly referred to pre-menstrual tension; (vii) reduced risk of development of many untoward symptoms (including dry vagina, peripheral flushing, depression etc) commonly associated in women with menopause. These and other complaints and diseases can be prevented or ameliorated in accordance with the invention.

The invention is therefore directed to a method for the prophylaxis or treatment of an animal, including a human, to combat conditions associated with phyto-oestrogen deficiency, which comprises administering to the animal an effective amount of phyto-oestrogen selected from the

isoflavones and/or the coumestans, ideally in concentrated form.

Preferably the phyto-oestrogens are derived from, or the phyto-oestrogen source comprises, the hull and/or hypocotyl of soyabean. However plants, other than soya, which contain significant amounts of isoflavones or coumestans can be used. Preferably also, the isoflavones are selected from a source rich in daidzein or genistein, most preferably daidzein, or their metabolites or derivatives. Particularly preferred is to use a product particularly rich in daidzein or its metabolite equol.

The invention also concerns formulations containing phyto-oestrogens selected from the isoflavones and/or coumestans. Such formulations containing the hull and/or hypocotyl of soyabeans are particularly preferred. Also preferred are formulations containing the isoflavones daidzein or genistein or their metabolites, or derivatives, especially equol.

The formulations may be a variety of kinds, such as nutritional supplements, pharmaceutical or veterinarial preparations, vitamin supplements, food additives or foods supplemented with the active phyto-oestrogens of the invention, liquid or solid preparations, including drinks, sterile injectable solutions, tablets, coated tablets, capsules, powders, drops, suspensions, or syrups, ointments, lotions, creams, pastes, gels, or the like. The formulations may be in convenient dosage forms, and may also include other active ingredients, and/or may contain conventional excipients, carriers and diluents. The inclusion of the subject phyto-oestrogens in herbal remedies and treatments is also a preferred part of the invention.

The invention is also directed to the amelioration, prevention, or of various conditions responsive to treatment with the phyto-oestrogen substances of the invention.

The invention is now described with reference to various examples.

Example 1 - preparation of soy phyto-oestrogen product. Soybeans were processed through a separation mill. Firstly, the beans are heated, so that the husk becomes brittle, then the mill removes the husk and splits the bean into the two cotyledons and the small-sized hypocotyl which separate from each other. Normal soybean separation mills discard the husks, and also separate and remove the small hypocotyl which can impart a bitter taste to the oil and soy-flour product from the soybeans.

For the purpose of the present invention these two "waste" products are collected separately, and optionally are processed further; for example by turning the husks and/or hypocotyl to a fine powder.

Further common processing steps can be used, if desired, to convert the husk or hypocotyl natural product or powder to capsule, tablet, liquid similar convenient form for ingestion. Otherwise the powder can be packaged as a convenient food additive or condiment, as occurs for other herbs and spices.

Example 2 - Effect of administering phyto-oestrogens to humans.

Tablets were prepared comprising 400 mg of whole soygerm (soy hypocotyls), and each tablet contained approximately 45 mg of isoflavones.

Two such tablets were taken daily by 18 volunteers for a period of 2 months. The effects of administering the soy phyto-oestrogen concentrate on blood cholesterol was determined by comparing the blood cholesterol levels in the volunteers before and after the experiment, with the levels of equol in urine.

Equol is the final metabolic product of one of the isoflavones, namely daidzein, in the body, and there is no other source of equol known other than from the metabolic reaction of daidzein. Therefore the presence of equol is directly the result of the soygerm tablets.

The results of the experiment are shown in the following Table. The total cholesterol is expressed as

mmol/litre and the equol level is expressed as mg per 24 hour urine volume. The results are presented as a range with the mean value shown in brackets. The individuals are grouped according to their ability to produce equol, in order to demonstrate the effects of administering the concentrated soy isoflavones.

	Pretreatment		Post-treatment	
Groups	Total cholesterol	Urinary equol	Total cholesterol	Urinary equol
Group 1 (n=7)	6.3-7.8 (7.2)	0-0.01 (0)	5.0-6.1 (5.6)	0.1-1.3 (0.9)
Group 2 (n=6)	5.0-6.2 (5.4)	0-0.01 (0)	44-5.3 (4.9)	0.3-2.4 (1.6)
Group 3 (N=5)	3.5-4.8 (4.0)	0-0.02 (0.01)	3.4-4.9 (4.0)	1.5-12.8 (6.8)

The pretreatment levels show that the average western diet contains little or no isoflavone, because of the very low amount of equol present. After the two months of treatment, appreciable equol levels are detected.

The results provide support for the ability of soygerm to lower blood cholesterol levels, due to the strong inverse correlation between an individual's baseline blood cholesterol level and the ability to metabolise daidzein to equol. That is, the greater is the person's ability to metabolise equol, then the lower is the cholesterol level.

The Table indicates that individuals with low baseline cholesterol levels and who are very efficient metabolizers of daidzein to equol, who make up Group 3, are likely to be obtaining sufficient dietary isoflavone to control their cholesterol, even though the amount of isoflavone they may be obtaining is small. As a result they show no response to their extra soygerm intake, in terms of the further lowering of either cholesterol levels. Whereas, those individuals in Groups 1 and 2 who have relatively less metabolic efficiency

in converting daidzein to equol, will respond to the additional dietary daidzein intake, with their cholesterol levels falling significantly.

Example 3 - Nutritional supplement products

(a) Finely ground soy hypocotyls is combined with Silybum marianum, vitamin B3 nicotinic acid) and Dandelion, to produce a natural health product to assist in controlling and lowering cholesterol levels in humans.

(b) Finely ground soy hypocotyls and husk is combined with some or all of the following herbs and vitamins, to prepare a natural health product for treating menopause: Dong quai, Vitex agnus castus, Sage, and Vitamin E.

(c) Finely ground soy hypocotyls and husk is combined with some or all of the following herbs and vitamins, to prepare a natural health product for treating pre-menstrual tension (PMT) and period problems in women: Black Haw, Dong quai, Vitex agnus castus, Calcium, Magnesium, B group vitamins, Vitamin E and Folic acid.

(d) Finely ground soy hypocotyls and husk is combined with some or all of the following herbs and vitamins, to prepare a natural health product for treating cancer: Betacarotene, Vitamin C, Vitamin E, Silybum marianum, Ginkgo and Bioflavonoids.

DATED this 19th day of May, 1992.

GRAHAM EDMUND KELLY
By His Patent Attorneys
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PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 31/35 — A1

(11) International Publication Number: WO 94/23716

(43) International Publication Date: 27 October 1994 (27.10.94)

(21) International Application Number: PCT/US94/04189

(22) International Filing Date: 15 April 1994 (15.04.94)

(30) Priority Data:
08/049,006 16 April 1993 (16.04.93) US

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

(57) Abstract

A method is provided for preventing or treating symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of an isoflavonoid. The invention also features a therapeutic dietary product, containing isoflavonoids, for preventing or treating symptoms of conditions resulting from reduced or altered levels of endogenous estrogen.

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METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Background of the Invention

The present invention relates to therapies for the prevention and treatment of menopausal and premenstrual symptoms.

It has long been recognized that the sharp reduction in endogenous estrogen levels which occurs prior to menopause causes a variety of unpleasant symptoms, e.g., hot flashes, nausea, nervousness, and malaise. Currently, the symptoms of menopause are treated by estrogen replacement therapy, which has recently been shown to increase the risk of certain types of cancer, such as endometrial cancer and breast cancer. Changes in levels of endogenous estrogen may also be responsible for "premenstrual syndrome", a condition occuring in younger women prior to menstruation. Premenstrual symptoms are treated with a variety of hormonal and nonhormonal therapies, which may cause side effects. Safer and more effective therapies for both conditions continue to be sought.

Summary of the Invention

The inventors have found that isoflavonoids, which are constituents of soy beans and other plants, effectively reduce the symptoms of conditions which are caused by reduced or altered levels of endogenous estrogen, e.g., menopause, and premenstrual syndrome. Without being bound by any theory, it is believed that the isoflavonoids bind to estrogen receptors, and thus exert an estrogenic response. These compounds, which are present naturally in soy-based and other plant-based foods, are safe and cause no significant side-effects. Isoflavonoids which may be administered according to the invention include genistein, daidzein, Biochanin A,

formononetin, O-desmethylangolensin, and equol; these may be administered alone or in combination.

Accordingly, in one aspect, the invention features a method of preventing or treating the symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of at least one isoflavonoid. The isoflavonoid may be administered in any suitable form, e.g., in the form of a plant extract rich in isoflavonoids or in the form of a purified or synthesized isoflavonoid.

In another aspect, the invention features a therapeutic dietary product for preventing or treating symptoms resulting from reduced or altered levels of endogenous estrogen. The dietary product preferably includes a soy extract containing enriched isoflavonoids, provided in a palatable food carrier, e.g., a confectionary bar, biscuit, cereal or beverage.

Other features and advantages of the invention will be apparent from the Description of the Preferred Embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Isoflavonoids are naturally occurring substances, found primarily in soy beans. These compounds are also found in lower concentrations in many other plants. Isoflavonoids can thus be administered to a patient by placing the patient on a diet containing high levels of soy-based food products, e.g., tofu, miso, soybeans, aburage, atuage and koridofu, or other plant products rich in isoflavonoids.

These products may not be readily available in all geographic regions (most of these foods are served predominantly in Japan), and are not be palatable to many women, particularly those accustomed to Western-style food.

Accordingly, an isoflavonoid-containing fraction can be extracted from a soy or plant product. It is preferred that the isoflavonoids be extracted and concentrated from soy bean or soy powder. Isoflavonoids are also available commercially in substantially pure form. The concentrated isoflavonoid is preferably included in a food carrier to form a dietary product. Any type of palatable carrier may be used, but, as the isoflavonoid concentrate has a strong flavor, it is preferred that the carrier include suitable flavorings to impart a different, more palatable flavor. The dietary product may be any type of food product, e.g., a confectionary bar, biscuit, cereal or beverage.

It is preferred that the dietary product contain at least 30 mg/serving total isoflavonoids. The isoflavonoid concentrate included in the dietary product preferably includes a blend primarily comprised of genistein and daidzein. The concentrate typically also contains lower levels of other isoflavonoids. Most preferably, the dietary product contains from about 10 to 30 mg/serving, more preferably about 20 mg/serving of genistein, and from about 5 to 10 mg/serving, more preferably about 7 mg/serving of daidzein. Preferably, a dietary product containing the preferred dosage of isoflavonoids would be consumed at least once per day, preferably 1 to 2 times per day depending upon the severity of the woman's symptoms.

While it is preferred that the isoflavonoid be administered in the form of a dietary product, if desired the isoflavonoid could be administered, preferably in similar dosages, in medicament form, e.g., mixed with a pharmaceutically acceptable carrier to form a tablet, powder or syrup.

Example

The connection between diet and estrogen excretion was studied in Japanese women and men, and in a few children. The women's mean age was 50.4 (SD 18.0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. Isoflavonoid excretion in the urine was measured in a group of three men, three women, and three children living in Kyoto and consuming the traditional diet. We found a very high excretion of isoflavonoids in the urine of these subjects. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15.5 umol per 24h in a man) and two other isoflavonoids, daidzein and equol (Table 1). All these compounds bind to estrogen receptors and have weak estrogenic activity. The excretion of the isoflavonoids in urine of the Japanese women was much higher than previously determined levels in American and Finnish women (Table 1). Excretion was high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than the levels of endogenous estrogens excreted by normal omnivorous women consuming a western or oriental diet (Table 1).

The excretion of the isoflavonoids in urine was associated with intake of soy products such as tofu, miso, aburage, atuage, koridofu, soybeans, and boiled beans.

It is known that Japanese women have a lower incidence of menopausal symptoms and premenstrual symptoms than the American and Finnish women.

Table 1

Urinary isoflavonoid or estrogen (nmol/day)	Japanese/ Oriental	American	Finnish
Genistein	3440(n=3)	. .	32.1(n=12)
Daidzein	2600(n=10)	216(n=21)	40.5(n=12)
Equol	2600(n=10)	62.8(n=21)	44.2(n=12)
Oestrone (postmenstrual)	4.48(n=9)	. .	4.48(n=10)
Oestradiol (postmenstrual)	0.76(n=9)	. .	0.94(n=10)
Oestriol (postmenstrual)	4.48(n=9)	. .	4.44(n=10)

CLAIMS

1. Use of an isoflavonoid in the preparation of a medicament for preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen.

2. The use of claim 1, wherein said isoflavonoid is selected from the group consisting of genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin and equol.

3. The use of claim 1 wherein said isoflavonoid is in a unit dosage of at least 30 mg.

4. The use of claim 1 wherein genistein and daidzein isoflavonoids are present in said medicament.

5. The use of claim 4 wherein said isoflavonoid comprises from about 10 to 30 mg genistein and from about 5 to 10 mg daidzein.

6. The use of claim 1 wherein said medicament is in the form of a dietary product.

7. The use of claim 6 wherein said dietary product contains at least 30 mg/serving of said isoflavonoid.

8. The use of claim 6 wherein said dietary product is a confectionery bar containing said isoflavonoid.

9. The use of claim 6 wherein said dietary product is a cereal containing said isoflavonoid.

10. The method of claim 6 wherein said dietary product is a biscuit containing said isoflavonoid.

11. The method of claim 6 wherein said dietary product is a beverage containing said isoflavonoid.

12. A dietary product for preventing or treating symptoms of menopause, premenstrual syndrome, or conditions resulting from reduced or altered levels of endogenous estrogen, comprising at least one isoflavonoid provided in a non-soy-based palatable food carrier.

13. The dietary product of claim 12 comprising genistein and daidzein isoflavonoids.

14. The dietary product of claim 12 wherein the food carrier is a confectionery bar.

15. The dietary product of claim 12 wherein the food carrier is a cereal.

16. The dietary product of claim 12 wherein the food carrier is a biscuit.

17. The dietary product of claim 12 wherein the food carrier is a beverage.

18. The dietary product of claim 12 wherein the food carrier contains an amount of the isoflavonoid which is effective in reducing the symptoms.

19. The dietary product of claim 18 comprising at least about 30 mg isoflavonoids per serving.

20. The dietary product of claim 13 wherein said dietary product comprises from about 10 to 30 mg/serving genistein and from about 5 to 10 mg/serving daidzein.

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PCT/US 94/04189

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BAR CODE LABEL

U.S. PATENT APPLICATION

08/049,006

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT
08/049,006	04/16/93	514	1205

APPLICANT

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CONTINUING DATA********
VERIFIED

REC'D 31 MAY 1994
WIPO PCT

FOREIGN/PCT APPLICATIONS********
VERIFIED

PRIORITY DOCUMENT

FOREIGN FILING LICENSE GRANTED 08/19/93 ***** SMALL ENTITY *****

STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS	FILING FEE RECEIVED	ATTORNEY DOCKET NO.
MA	0	22	2	$442.00	05495/003001

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TITLE

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application which is identified above.

By authority of the
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Date APR 25 1994 Certifying Officer

PATENT APPLICATION SERIAL NO. 08/049006

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

080 AT 05/03/93 08049006 1 201 377.00 CK

PTO-1556
(5/87)

APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

APPLICANT: SHERWOOD L. GORBACH, BARRY R. GOLDIN AND HERMANN ADELCREUTZ

"Express Mail" mailing label number RR880901164US

Date of Deposit April 16, 1993

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ATTORNEY DOCKET NO: 05495/003001

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Background of the Invention

The present invention relates to therapies for the prevention and treatment of menopausal and premenstrual symptoms.

It has long been recognized that the sharp reduction in endogenous estrogen levels which occurs prior to menopause causes a variety of unpleasant symptoms, e.g., hot flashes, nausea, nervousness, and malaise. Currently, the symptoms of menopause are treated by estrogen replacement therapy, which has recently been shown to increase the risk of certain types of cancer, such as endometrial cancer and breast cancer. Changes in levels of endogenous estrogen may also be responsible for "premenstrual syndrome", a condition occuring in younger women prior to menstruation. Premenstrual symptoms are treated with a variety of hormonal and nonhormonal therapies, which may cause side effects. Safer and more effective therapies for both conditions continue to be sought.

Summary of the Invention

The inventors have found that isoflavonoids, which are constituents of soy beans and other plants, effectively reduce the symptoms of conditions which are caused by reduced or altered levels of endogenous estrogen, e.g., menopause, and premenstrual syndrome. Without being bound by any theory, it is believed that the isoflavonoids bind to estrogen receptors, and thus exert an estrogenic response. These compounds, which are present naturally in soy-based and other plant-based foods, are safe and cause no significant side-effects. Isoflavonoids which may be administered according to the invention include genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin, and equol; these may be administered alone or in combination.

Accordingly, in one aspect, the invention features a method of preventing or treating the symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of at least one isoflavonoid. The isoflavonoid may be administered in any suitable form, e.g., in the form of a plant extract rich in isoflavonoids or in the form of a purified or synthesized isoflavonoid.

In another aspect, the invention features a therapeutic dietary product for preventing or treating symptoms resulting from reduced or altered levels of endogenous estrogen. The dietary product preferably includes a soy extract containing enriched isoflavonoids, provided in a palatable food carrier, e.g., a confectionary bar, biscuit, cereal or beverage.

Other features and advantages of the invention will be apparent from the Description of the Preferred Embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Isoflavonoids are naturally occurring substances, found primarily in soy beans. These compounds are also found in lower concentrations in many other plants. Isoflavonoids can thus be administered to a patient by placing the patient on a diet containing high levels of soy-based food products, e.g., tofu, miso, soybeans, aburage, atuage and koridofu, or other plant products rich in isoflavonoids.

These products may not be readily available in all geographic regions (most of these foods are served predominantly in Japan), and are not be palatable to many women, particularly those accustomed to Western-style food.

Accordingly, an isoflavonoid-containing fraction can be extracted from a soy or plant product. It is preferred

that the isoflavonoids be extracted and concentrated from soy bean or soy powder. Isoflavonoids are also available commercially in substantially pure form. The concentrated isoflavonoid is preferably included in a food carrier to form a dietary product. Any type of palatable carrier may be used, but, as the isoflavonoid concentrate has a strong flavor, it is preferred that the carrier include suitable flavorings to impart a different, more palatable flavor. The dietary product may be any type of food product, e.g., a confectionary bar, biscuit, cereal or beverage.

It is preferred that the dietary product contain at least 30 mg/serving total isoflavonoids. The isoflavonoid concentrate included in the dietary product preferably includes a blend primarily comprised of genistein and daidzein. The concentrate typically also contains lower levels of other isoflavonoids. Most preferably, the dietary product contains from about 10 to 30 mg/serving, more preferably about 20 mg/serving of genistein, and from about 5 to 10 mg/serving, more preferably about 7 mg/serving of daidzein. Preferably, a dietary product containing the preferred dosage of isoflavonoids would be consumed at least once per day, preferably 1 to 2 times per day depending upon the severity of the woman's symptoms.

While it is preferred that the isoflavonoid be administered in the form of a dietary product, if desired the isoflavonoid could be administered, preferably in similar dosages, in medicament form, e.g., mixed with a pharmaceutically acceptable carrier to form a tablet, powder or syrup.

Example

The connection between diet and estrogen excretion was studied in Japanese women and men, and in a few

children. The women's mean age was 50.4 (SD 18.0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. Isoflavonoid excretion in the urine was measured in a group of three men, three women, and three children living in Kyoto and consuming the traditional diet. We found a very high excretion of isoflavonoids in the urine of these subjects. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15.5 umol per 24h in a man) and two other isoflavonoids, daidzein and equol (Table 1). All these compounds bind to estrogen receptors and have weak estrogenic activity. The excretion of the isoflavonoids in urine of the Japanese women was much higher than previously determined levels in American and Finnish women (Table 1). Excretion was high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than the levels of endogenous estrogens excreted by normal omnivorous women consuming a western or oriental diet (Table 1).

The excretion of the isoflavonoids in urine was associated with intake of soy products such as tofu, miso, aburage, atuage, koridofu, soybeans, and boiled beans.

It is known that Japanese women have a lower incidence of menopausal symptoms and premenstrual symptoms than the American and Finnish women.

Other embodiments are within the claims.

Table 1

Urinary isoflavonoid or estrogen (nmol/day)	Japanese/ Oriental	American	Finnish
Genistein	3440(n=3)	. .	32.1(n=12)
Daidzein	2600(n=10)	216(n=21)	40.5(n=12)
Equol	2600(n=10)	62.8(n=21)	44.2(n=12)
Oestrone (postmenstrual)	4.48(n=9)	. .	4.48(n=10)
Oestradiol (postmenstrual)	0.76(n=9)	. .	0.94(n=10)
Oestriol (postmenstrual)	4.48(n=9)	. .	4.44(n=10)

CLAIMS

1. A method of preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen, said method comprising administering to the woman an effective amount of an isoflavonoid.

2. The method of claim 1, wherein said isoflavonoid is selected from the group consisting of genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin and equol.

3. The method of claim 1 wherein said isoflavonoid is administered in a dosage of at least 30 mg.

4. The method of claim 3 wherein said isoflavonoid is administered in said dosage at least once per day.

5. The method of claim 1 wherein genistein and daidzein isoflavonoids are coadministered.

6. The method of claim 5 wherein said isoflavonoid comprises from about 10 to 30 mg genistein and from about 5 to 10 mg daidzein.

7. The method of claim 1 wherein said isoflavonoid is administered in the form of a dietary product.

8. The method of claim 7 wherein said dietary product contains at least 30 mg/serving of said isoflavonoid.

9. The method of claim 7 wherein said dietary product is a confectionery bar containing said isoflavonoid.

10. The method of claim 7 wherein said dietary product is a cereal containing said isoflavonoid.

11. The method of claim 7 wherein said dietary product is a biscuit containing said isoflavonoid.

12. The method of claim 7 wherein said dietary product is a beverage containing said isoflavonoid.

13. The method of claim 7 wherein said dietary product is consumed by said woman at least once per day.

14. A dietary product for preventing or treating symptoms of menopause, premenstrual syndrome, or conditions resulting from reduced or altered levels of endogenous estrogen, comprising at least one isoflavonoid provided in a non-soy-based palatable food carrier.

15. The dietary product of claim 14 comprising genistein and daidzein isoflavonoids.

16. The dietary product of claim 14 wherein the food carrier is a confectionery bar.

17. The dietary product of claim 14 wherein the food carrier is a cereal.

18. The dietary product of claim 14 wherein the food carrier is a biscuit.

19. The dietary product of claim 14 wherein the food carrier is a beverage.

20. The dietary product of claim 14 wherein the food carrier contains an amount of the isoflavonoid which is effective in reducing the symptoms.

21. The dietary product of claim 20 comprising at least about 30 mg isoflavonoids per serving.

22. The dietary product of claim 15 wherein said dietary product comprises from about 10 to 30 mg/serving genistein and from about 5 to 10 mg/serving daidzein.

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Abstract of the Disclosure

A method is provided for preventing or treating symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of an isoflavonoid. The invention also features a therapeutic dietary product, containing isoflavonoids, for preventing or treating symptoms of conditions resulting from reduced or altered levels of endogenous estrogen.

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PATENT
ATTORNEY DOCKET NO: 05495/003001

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS, the specification of which

is attached hereto.

☒ was filed on APRIL 16, 1993 as Application Serial No. 08/049,006
and was amended on _____.

☐ was described and claimed in PCT International Application No.
filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: William E. Booth, Reg. No. 28,933; Barry E. Bretschneider, Reg. No. 28,055; Paul T. Clark, Reg. No. 30,162; Willis M. Ertman, Reg. No. 18,658; David L. Feigenbaum, Reg. No. 30,378; John W. Freeman, Reg. No. 29,066; Timothy A. French, Reg. No. 30,175; Alan H. Gordon, Reg. No. 26,168; Gilbert H. Hennessey, Reg. No. 25,759; Charles Hieken, Reg. No. 18,411; Robert E. Hillman, Reg. No. 22,837; G. Roger Lee, Reg. No. 28,963; Steven E. Lipman, Reg. No. 30,011; Gregory A. Madera, Reg. No. 28,878; Ralph A. Mittelberger, Reg. No. 33,195; Ronald E. Myrick, Reg No. 26,315; Frank P. Porcelli, Reg. No. 27,374; Eric L. Prahl, Reg. No. 32,590; Alan D. Rosenthal, Reg. No. 27,833; John M. Skenyon, Reg. No. 27,468; Michael O. Sutton, Reg. No. 26,675; Rene D. Tegtmeyer, Reg. No. 33,567; John N. Williams, Reg. No. 18,948; Gary A. Walpert, Reg. No. 26,098; Charles C. Winchester, Reg. No. 21,040; and Celia H. Ketley, Reg. No. 33,524.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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ATTORNEY DOCKET NO. 05495

Applicant or Patentee: SHERWOOD L. GORBACH ET AL.
Serial or Patent No.: 08/049,006
Filed or Issued: APRIL 16, 1993
For: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: TUFTS UNIVERSITY SCHOOL OF MEDICINE
Address of Organization: BOSTON, MA
Type of Organization: UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

[X] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE:)
(CITATION OF STATUTE:)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE:)
(CITATION OF STATUTE:)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1. for purposes paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the in tion entitled METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS by inventor(s) SHERWOOD L. GORBACH, BARRY R. GOLDIN, and HERMAN ADLERCREUTZ described in

[ ] the specification filed herewith.
[X] application serial no. 08/049,006, filed APRIL 16, 1993.
[ ] patent no. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name: _____

Address: _____

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I by declare that all statements made herein of my own knowledge are true and that all statements made on information and be...f are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name: ROBERT GARRISON

Title: DIRECTOR, PATENTS & LICENSING

Address: TUFTS UNIVERSITY, PACKARD HALL, MEDFORD, MA 02155

Signature: _____ Date: 1/6/93

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Herbal Help to Avoid Menopause Symptoms

by Nancy Beckham

The aim of this article is to provide information on non-harmful ways of overcoming the problems of menopause.

The information given may also be applicable to women who already have osteoporosis or for younger women who have had their ovaries removed, however these two categories of women should seek professional guidance.

SOME STATISTICS

Twenty-five per cent of women in the 45–55 age range have no menopausal symptoms. Of the 75 per cent who have problems, the following is a breakdown of the symptoms:

Flushing and sweats	80%
Lethargy	70%
Nervous problems such as anxiety, depression, irritability	70%
Reduced sex drive	65%
Insomnia	60%

Other symptoms include hair and skin changes, poor memory and lack of concentration, headaches, dry vagina, pain during intercourse, loss of confidence, loss of femininity and urinary symptoms. Of course, not all of these are necessarily linked to low oestrogen levels and could be related to dietary and lifestyle factors and the 'normal' aging process. After middle age, men also find they have less energy, a lower sex drive and generally sleep less.

Osteoporosis is the most serious problem associated with menopause because as much as 50 per cent of total bone mass may be lost by the time a woman reaches 70 years of age, which means that bones can fracture easily and healing may be prolonged. This disease does not affect all regions – it is rare in African Negroes and there are areas where it affects more men than women. In Australia, it is estimated that about 25 per cent of post-menopausal women have osteoporosis. I will deal with this in detail next issue.

What happens when the ovaries stop functioning?

The major factor is the lowered production of oestrogen. However, this hormone can be produced in other glands, such as the adrenals, but obviously, in many women, this does not occur quickly enough or in sufficient quantities. Basically, the hormonal system works on a feedback system; when the circulating levels are high, a chemical 'messenger' instructs our endocrine system not to produce any more of that particular hormone. Obviously, if we flood our system with a hormonal drug, the messages to our endocrine system will be to stop production. This may explain why some women do not menstruate for varying periods when they stop taking the Pill.

Most menopausal-age women will need to give their bodies as much assistance as possible so that sufficient oestrogen is produced to offset flushing and other symptoms. In nearly every case, this apparently happens over a period of time as the obvious symptoms gradually lessen and disappear. This added function of the adrenals may partly explain why some women have difficulty handling stress at menopause.

The controversial topic of hormonal replacement therapy will be discussed in detail later but in view of this feedback mechanism, it may not be wise to completely dampen the corrective biological function which already exists.

I am not suggesting that we can avoid the inevitability of aging, but I can't accept that whatever power 'designed' us also programmed that we were predestined to suffer a range of serious problems after middle age. We must be doing something wrong or there must be non-harmful methods of preventing the symptoms.

OESTROGENS IN FOODS AND HERBS AND HOW TO USE THEM

Since the 1920s over 50 different species of plant have been found to contain oestrogenic substances. Most of the published research papers relate to the effects on animals, particularly in respect of clovers and alfalfa (lucerne) causing infertility in farm animals. It so happens that a number of these plants have been used by herbalists over the centuries and this 'tested' use on humans has verified the hormonal effect. The tiny quantities of oestrogens in plants are extremely weak compared to pharmaceutical hormones but many women alleviate symptoms through sensible dietary changes.

Some of these oestrogen-containing plants are:

Alfalfa

The sprouts are particularly recommended as they have the added advantage of being very low in calories, readily available in shops or you can make your own, palatable in salads or sandwiches, mildly alkaline and rich in nutrients, especially calcium and potassium.

Alfalfa sprouts are somewhat controversial at the moment as the Gerson Institute in Mexico has reported that they suppress the immune system and aggravate conditions such as rheumatoid arthritis and systemic lupus erythematosus (SLE). The origin of this report was that two women had seemingly reactivated SLE following the ingestion of 10 and 15 alfalfa tablets per day. A particular constituent, L-canavanine, was extracted from alfalfa and when this isolated extract was given to susceptible animals, SLE was reactivated.

My own view is that, as SLE is a condition which has relative periods of aggravation and remission, it would be difficult to 'blame' one particular dietary item. Over 25 pharmaceuticals exacerbate the disease, isolated extracts of plants are in the nature of drugs and one would therefore expect side-effects and, most importantly, if this type of criterion were applied to almost any edible food, there would be very little left for us to eat. However, it may be prudent for sufferers of SLE to avoid alfalfa, all sprouted seeds and legumes, such as lentils, because these also contain the suspected irritant.

Red Clover

This is commonly sold as a herbal tea but I suggest you buy the seeds and sprout them. Please do not pick the clover yourself because the medicinal species, called *Trifolium pratense*, is difficult to distinguish from some non-edible clovers. Red clover is also used by professional herbalists for skin and respiratory conditions.

Sage

The common garden sage or red sage is used. It is better to grow your own but sage can be difficult to cultivate, mainly because it prefers a light, well-drained soil.

Sage has been used for centuries for excess sweating and heat, and scientific research has confirmed its oestrogen content. The best way to prepare it as a remedy for flushing is to soak two

tablespoons of finely chopped fresh leaves (or one tablespoon dried) in 500 mL tepid water with the juice of a lemon. Leave it stand in a covered jar overnight. Strain and keep in the fridge. In severe cases you would drink the whole quantity throughout the day; where the symptoms are relatively minor, then the 500 mL could be spread over two or three days. To make it more palatable, you could mix it with a fruit or vegetable juice, or add in some crushed fennel or aniseed. The high dose may need to be used for up to four weeks, then it could be gradually reduced to a cup per day.

The old sage you have had in your cupboard since two Christmases ago probably no longer retains any therapeutic properties; good-quality dried sage will still have a reasonably good colour and its characteristic strong odour.

Parsley

This common culinary herb has oestrogen-like activity and I would suggest a handful per day; it may not be wise to use larger quantities because of the myristicin and apiol content.

Aniseed

Use the crushed seeds as a herbal tea or in cooking, for example in home-made bread. The seeds could also be added to apple cider vinegar and used in salad dressings. Finely chopped fresh leaves can be added to salads, steamed vegetables and soups. Aniseed is also helpful for minor digestive problems and coughs.

Fennel

The seeds and finely chopped fresh leaves can be used in a similar way to aniseed. There is one species of fennel (Florence) which develops a bulb-like base and this may be used like celery or lightly steamed. Some greengrocers call it aniseed root. Wild fennel is a common weed and, although the seeds and leaves could be used, this plant is often contaminated with environmental pollutants.

Similar culinary herbs, such as dill and caraway, probably contain mild oestrogen-like substances.

Hops

Some health-food stores sell dried hops. It is somewhat bitter, which may also stimulate the digestive function, but the tea should be made quite weak otherwise it is not very palatable. An important feature of hops is that it has a sedative function and for this reason herbal extracts of hops are not used by professional herbalists where there is depression. Many people find that hops helps with insomnia – a herbal pillow using dried hops can be quite beneficial.

The hormonal content of hops has been verified; females harvesting it have altered menstrual periods solely from external contact. I am not sure whether or not beer, after all the processing, would retain any oestrogenic properties.

Soya Beans

Sprouts are the best way to have these, particularly as the sprouting dramatically increases the oestrogen content. However, they are quite difficult to sprout because they go mouldy and smelly if not washed and drained thoroughly and often. They are amazingly tasty but wait until you have learned to sprout alfalfa and mung beans before trying them. I add soya bean sprouts to salads or use them to thicken soups and casseroles.

Dried soya beans need to be soaked and cooked for a long time and they are probably best used in soups and casseroles but there are many ways of preparing them to make them more appetising. They are cheap, an excellent protein when combined with a grain and have other benefits, such as being protective against atherosclerosis.

If you don't normally eat dried beans then you must start with small quantities, soaked overnight and very well cooked, otherwise you will probably have severe abdominal colic and flatulence. This is partly because certain enzymes have to be activated to handle such foods and your digestive system needs time to adjust.

Soya beans are also a leguminous plant so, theoretically, could have the same cautions as indicated under alfalfa.

Dried red beans and common green beans are also mildly oestrogenic so could be included in the diet on a regular basis.

There is some evidence that all young sprouts, including sprouted grains and legumes, have oestrogenic properties and, as sprouts are cheap, pesticide and chemical free, rich in nutrients and low in calories, I recommend that you learn how to do your own and have at least one cup per day if you are a menopause-age female. You can buy small paperback books giving you basic instructions for sprouting and use jars, so the starting equipment is not expensive.

Some words of warning: When using seeds to sprout, never use those that are intended for agricultural purposes because they would have been treated with fungicides or other chemicals which are potentially dangerous.

Fenugreek

Contains precursors of progesterone, another female hormone commonly deficient in menopausal women. Unfortunately, the curry-like smell is readily excreted through the skin but this is not so noticeable if the seeds are sprouted.

There are other herbal and naturopathic remedies for menopausal problems but these are not normally available at retail outlets so you would need to visit a practitioner to obtain these. As with most health problems, there are mild symptoms which require no treatment or simple home remedies; then there are other instances where professional naturopathic advice is helpful and appropriate; and, finally, there are severe cases which require medical diagnosis and treatment.

OTHER SUGGESTIONS

Potassium sulphate, used in the form of tissue salts, may be helpful for flushing. Use the dosage on the label, but take double the dose for the first week.

Vitamin E has also alleviated some cases of flushing; furthermore, a study on rats showed that a vitamin E deficiency leads to lower bone weight. As this vitamin has benefits to the cardiovascular system, a supplement of 500 iu per day would do no harm and may give marked benefits.

Cigarette smoking tends to bring on early menopause and is not recommended for this and other well-publicised reasons.

Low-calorie diets are not recommended for a number of reasons which are given later, but one important factor is that fat cells are able to convert hormones from the adrenal glands into oestrogen. Although modern women, including myself, don't want or need to be obese, it may be that 'nature' intended us to carry more weight as we age.

Readers may be interested in a few snippets from some of the research material which I have collected:

Journal of Food Protection, Vol. 42, July 1979, states that 'human exposure to dietary oestrogens is below physiological levels ... but the possibility of metabolic alterations to more or less active forms should not be ignored since effects of this kind have been demonstrated in experimental animals.'

Oestrogenic Constituents of Forage Plants, E.M. Bickoff, Review Series 1/1968, published by the Commonwealth Bureau of Pastures and Field Crops, Hurley, Berkshire, reports that 'the classical infertility syndrome in ewes is associated with the cumulative effects of exposure to oestrogenic feeding for six months or longer, but short-term exposure has also caused reproductive disturbances.'

The effect of oestrogenic plant substances is judged by changes in the anatomy of animals, for example increased uterine and ovarian weight, teat length and thicker vaginal skin.

A particularly interesting piece of research has shown that genistein, a weak plant oestrogen, is able to displace oestradiol from receptors in the tissues which could explain why some herbal

Continued on next page

Herbal Help to Avoid Menopause Symptoms

remedies are traditionally used for 'balancing' hormones.

Both the liver and the kidneys have a capacity for converting and deactivating different types of oestrogens; there are also other regulatory mechanisms, such as the prostaglandins.

HORMONE REPLACEMENT THERAPY

Although mainstream medical opinion supports hormone replacement therapy, it is somewhat controversial. Few disagree with the fact that it prevents the worsening of osteoporosis in postmenopausal females but the main disadvantage is in the potential side-effects. The current scientific thinking is that if both oestrogen and progestogen are taken together there is less risk of cancer. I am using Depo-Provera and Premarin as examples because a lady I saw recently had been prescribed these for menopausal flushing and she had written to the two manufacturers for information. The manufacturer of Premarin sent back 22 pages of reports and a book, all giving glowing testimonials and other information but only a few fragments about risks. The manufacturer of Depo-Provera sent a copy of the official package insert, with all the contraindications and side-effects, along with a letter which stated that 'as adjunct to cyclic oestrogen therapy (including Premarin) Depo-Provera is not recommended but it is still definitely used by medical practitioners'.

Depo-Provera

The contraindications and warnings for this drug include thromboembolic disorders (clotting), cerebral apoplexy (stroke), impaired liver function, undiagnosed vaginal bleeding and cerebrovascular (heart and circulatory) disorders. 'In cases of partial or complete loss of vision, sudden onset of proptosis (displacement of an organ), double vision, migraine associated with retinal vascular lesions, medication should be withdrawn.' The drug caused malignant breast nodules in animals. Other problems include fluid retention, breakthrough bleeding and depression. It is not approved for contraception because of unresolved questions relating to its safety for this purpose. Clinically, it is said that the drug is well tolerated although animal studies show that it possesses adrenocorticoid activity and female masculinisation.

Premarin

This drug is 'probably effective for oestrogen deficiency-induced osteoporosis only when used in conjunction with other important measures such as diet, calcium, physiotherapy and good general health-promoting measures'. The contraindications and cautions include impaired liver function, breast cancer (with some exceptions), thromboembolic disorders, undiagnosed abnormal genital bleeding and pregnancy. It should not be given to women with recurrent chronic mastitis and abnormal mammograms. It should only be prescribed following a complete breast and pelvis examination. Because the body produces variable amounts of oestrogen, relative overdosage may occur which could lead to uterine bleeding, painful, swollen breasts and fluid retention. Drug oestrogens need to be used with care in cases of epilepsy, migraine, asthma, heart or kidney disease. Side-effects include nausea, abdominal cramps and bloating, breast tenderness, changes in body weight, allergic rash and gallbladder complications.

When the two are prescribed together, there is often a monthly bleed.

If you are one of those people who believe that 'it would not be allowed by the government if there were risks involved', I suggest you read the information for yourself in *Mims Annual* which is available at most libraries.

A report in the *New England Journal of Medicine*, 19 June 1986, states that 'oral administration of oestrogens is inefficient, produces a non-physiologic pattern of breakdown products and increases undesirable levels of certain liver proteins'. The article examines the use of transdermal oestrogen therapy (skin patches) which would provide levels equivalent to those produced naturally. However, according to *Modern Medicine in Australia*, August 1986, transdermal oestrogen does not prevent osteoporosis.

Mainstream medical reports recommend oestrogen, used in conjunction with progesterone, as being the most effective therapy for preventing fractures, and a number of international experts suggest that all women should be considered probable victims and that hormone replacement therapy should begin soon after menopause in women, unless there are specific contraindications. The reasons for this are that at this stage there are no practical methods of clearly preselecting those at risk and tests show that it is the only therapy that clearly prevents further bone deterioration.

The critics point out that although adding progesterone to the therapy probably reduces the risk of endometrial cancer, there is insufficient data about the long-term safety or benefits – it only treats the symptoms and is unlikely to help the body re-establish its own state of internal harmony.

A pamphlet issued by the NSW Department of Health states that you should 'think carefully about whether or not you want hormone replacement therapy'. Some of my patients have understood from their medical consultations that hormone replacement therapy prevents cancer and cardiovascular disease, which is not true. Patients who are under this impression should discuss this matter with their practitioners.

The fact that a substance can prevent further bone breakdown does not necessarily mean that lack of it caused the problem, just as Valium helps you sleep but lack of it did not cause your insomnia.

In any event, most experts agree that there are clearly individuals who should not undertake hormonal replacement therapy and such people can use the non-harmful suggestions in this article.

No one enjoys being wrinkled and cranky but it is generally conceded that hormonal replacement therapy is not appropriate for cosmetic and emotional purposes.

Next issue: Osteoporosis and calcium requirements.

Nancy Beckham is the author of The Family Guide to Natural Therapies, *Greenhouse Publications, recommended retail price, $24.95.*

• *Reproductive Toxicology Review*

REPRODUCTIVE AND GENERAL METABOLIC EFFECTS OF PHYTOESTROGENS IN MAMMALS

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Key Words: Phytoestrogens, Mammalian reproduction, Reproductive hormones, Gonadal steroids, Luteinizing hormone, General Metabolism, Ovarian function, Reproductive neuroendocrinology.

INTRODUCTION

Historically, phytoestrogens were first investigated when it was noted that ewes that grazed Australian clover pastures for prolonged periods of time became sterile. It was found that the active agents in the clover that precipitated sterility were estrogenic (1). Later a similar phenomenon was observed to occur in the California quail during dry years, when phytoestrogen concentrations in available forage were increased (2).

Phytoestrogens are defined as plant substances that are structurally and functionally similar to the gonadal steroid 17β-estradiol (E_2) or that produce estrogenic effects (3). There are three main groups of nonsteroidal dietary estrogens. Phytoestrogens include the isoflavones (i.e., genistein, genistin, daidzein, biochanin A, formononetin, and pratensein) and the coumestans (i.e., coumestrol and 4'-o-methylcoumestrol). Mycoestrogens of the resorcylic acid lactone group (i.e., zearalenone and zearalenol) are also commonly found (4). The structural similarity between these substances, endogenous mammalian estrogens (E_2 and estrone), and potent synthetic estrogens (diethylstilbestrol) have been studied (Figure 1). Isoflavones, the monocarboxylic derivatives of the 15-C flavones, and coumestans contain central structures of 15 carbons. Both of these groups are derivatives of 3-phenylchroman (Figure 1) and thus may be considered a single family of compounds (5). The fungal resorcylic acid lactones and endogenous estrogens possess central structures of 17 carbons.

The similarity among these compounds has led investigators to study the possibility that phytoestrogens might act on physiological processes and behavioral patterns to alter reproductive performance (3). If reproductive effects occur, then these compounds might have a role in the evolutionary success of herbivores, perhaps making the difference between survival and extinction for some species. It is possible that phytoestrogens, through mimicry of endogenous animal estrogens, function as defensive substances by which plants diminish the fertility of herbivores which feed on the plants (6). In effect, the phytoestrogens may be seen as one of the many variables determining animal fitness for survival. This argument is supported by noting that animal species differ in their sensitivity to phytoestrogens (7). Some species are relatively resistant to the estrogenic effects of these compounds, while others may suffer sterility as a result of prolonged ingestion of phytoestrogens. We have hypothesized that phytoestrogen-induced physiologic and behavioral effects in mammals are significant factors in the reproductive and therefore evolutionary success of the consuming species. We have initiated our analysis of this broad hypothesis by reviewing the available data relevant to the reproductive and general metabolic effects of phytoestrogens in mammals.

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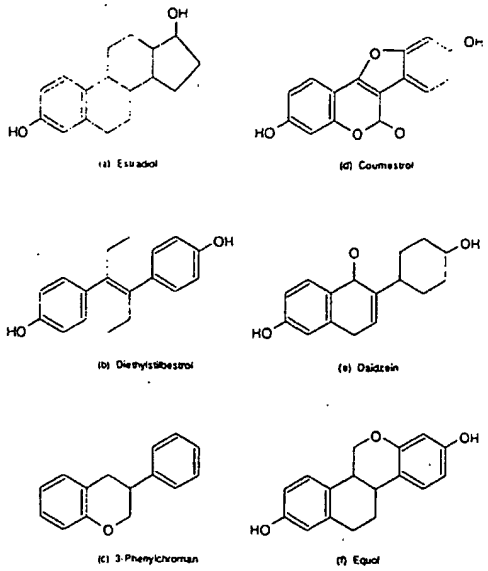

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f the isoflavone group.
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y Origin. In: Estrogens
achlan, ed. New York:
Elsevier Press, 1985; 69–85.)

PHYTOESTROGEN EXPOSURE

Sources of phytoestrogens

Phytoestrogens are produced by numerous Leguminosae and grasses, including many plants commonly consumed by man and livestock (Table 1). The estrogenic components are found in differing amounts in all parts of the plant, including the seeds, the flowers, the leaves, the roots, and the fruits. Concentrations in each tissue depend on plant type (4,8).

Of particular interest regarding possible human exposure is the presence of phytoestrogens in marijuana and coffee. It had long been suspected that the estrogenic effects of marijuana were due to Δ^9-tetrahydrocannabinol (THC), the major psychoactive compound. Smoking of marijuana significantly suppresses luteinizing hormone (LH) levels

Table 1. Some common plants that contain estrogenic substances

Alfalfa	Coffee	Oats	Rice
Anise	Date Palm	Orchard grass	Rye
Apple	Fennel	Palmetto grass	Sage
Barley	French Beans	Parsley	Sesame
Blue grass	Garlic	Peas	Soybean
Carrot	Green Beans	Pomegranate	Soya sprouts
Cherry	Hops	Potato	Wheat
Clovers	Liquorice	Rape	Yeast
	Marijuana	Red Beans	

during the human menstrual cycle and shortens both the menstrual cycle and the luteal phase (9). Since these results agree with observations in ovariectomized rhesus monkeys injected intramuscularly (i.m.) with THC, it was assumed that the menstrual cycle effects of smoke inhalation would be exclusively due to the THC content of the smoke (10). However, crude marijuana extract and condensed marijuana smoke compete with estradiol for estrogen receptors in the uterus of rats, while in vitro studies detected no binding of cannabinoids to estrogen receptors (11). These findings show that marijuana contains estrogenic substances that may be affecting reproductive processes via cannabinoid-independent mechanisms. Furthermore, apigenin, a derivative of flavonoid phytoestrogens found in crude marijuana, is a moderately potent inhibitor of estradiol binding to uterine estrogen receptors (11). Differentiation between the suppressive effect of THC on LH and the estrogenic effects of marijuana *per se* remains unclear.

Another plant product which is commonly ingested for pleasure rather than nutrition is coffee. Like marijuana, coffee contains weakly estrogenic constituents, evidenced by the estrogenic effects of increased uterine-to-body weight ratio and total uterine protein content following administration of coffee extracts by gavage (12). Ultraviolet absorbance spectroscopy suggests that whatever this active compound may be, it does not belong to one of the three major classes of dietary estrogens (e.g., flavonoids, coumestans, or resorcylic acid lactones). Thus, coffee may contain an estrogen precursor that requires metabolic activation or a structurally unrelated estrogenic compound.

Metabolism, distribution, and clearance

The relative potency of a phytoestrogen depends upon the target tissue, functional state of the target tissue, the animal species involved, and the route and pattern of delivery. In addition, the fami-

lies of estrogenic compounds that occur in plants can be modified by metabolism within the herbivore or even by gut flora prior to uptake. Dietary isoflavone phytoestrogens undergo bacterial modification in the gastrointestinal tracts of animals to yield equol, a weak, nonsteroidal phytoestrogen (8,13,14). Following ingestion of estrogenic plants, a temporary 50- to 1000-fold increase in urinary equol takes place, while insignificant traces of the initially consumed phytoestrogens appear in the urine. Noteworthy is that the major urinary product following the consumption of genistein and biochanin A is p-ethyl phenol, and formononetin consumption yields both daidzein and equol as the major urinary products (4). Furthermore, gut microflora (14) convert daidzein to equol which in turn is absorbed and enters the enterohepatic circulation. Notably, it appears that not all people have the ability to convert other isoflavones to equol. This may be due to the absence of bacteria capable of the conversion of precursors to equol (as is the case in the sterile gut of newborns), the composition (subpopulations) of intestinal microflora present, the intestinal transit time, pH, or redox potential. These factors may be influenced by diet, host immunity, medication use, etc.

Receptor activity and interaction with endogenous estrogens

Phytoestrogens exhibit binding to endogenous estrogen receptors. Binding of phytoestrogens to estrogen receptors is supported by the finding that the larger the dose of phytoestrogen given an organism, the greater the displacement of bound tritiated (^{3}H) E_2 (15). It has also been reported that at very high dosages, all phytoestrogens exhibit more than 80% competitive binding to renal tumor cytosolic estrogen receptors (16). The structural requisites for estrogen receptor binding are met by phytoestrogens. For example, equol possesses a potency on the order of 10^{-3} the estrogenic activity of E_2 and contains a phenyl substituent also present in E_2 and in DES (Figure 1). The substituent considered to be a requirement for estrogenic activity is a hydroxyl group in the same position as the hydroxyl group in the benzene ring of E_2 (14). Another structural similarity which facilitates estrogen receptor binding activity of equol and other phytoestrogens is that the distance between C-3 and C-17 in E_2 is about equal to that between the two hydroxyls in equol.

Considering the large quantities of phytoestrogens ingested by many mammals including man, functionally significant estrogen receptor occupancy by phytoestrogens occurs. Since no phytoestrogen has receptor affinity equal to that of E_2 and the degree of DNA stimulation due to phytoestrogens appears to be substantially less than that evoked by E_2 (8), phytoestrogen actions could be either estrogenic or anti-estrogenic. In a relatively hypoestrogenic individual, receptor occupancy by weak (exogenous) estrogens would likely produce estrogenic effects, while in a normally estrogenized individual, large amounts of weak estrogens might diminish the effective estrogenic activity by competition with E_2.

REPRODUCTIVE EFFECTS IN MAMMALS

Phytoestrogens have been shown to influence virtually every aspect of the mammalian reproductive process via effects on the morphology and physiology of reproductive organs and alteration of sexual behavior. The changes may be reversible or irreversible, depending on the duration and dose of exposure to the phytoestrogens.

Cervix

A pubertal pattern of cell differentiation has been noted in ewes rendered sterile by chronic ingestion of phytoestrogens (17). Among these changes, the cervix assumes a uterine pattern. Folds present in the cervix fuse, resulting in loss of cervical crypts, and the cells of the lamina propria become like those of the uterine stroma. Furthermore, glands having histochemical reactions reminiscent of uterine glands become plentiful in the cervix. Such an increase in abnormal glands may be responsible for the different composition which the cervical mucus takes in sheep with "clover disease." At low phytoestrogen dosage, the cervical mucus has a lower viscosity, not due to a higher water content, but rather due to a decreased concentration of glycoprotein — the component of mucus that affords its consistency. The level of glycoprotein seems to respond to the duration of exposure to the phytoestrogen rather to the dosage of the agent. This change in the cervical mucus compounds the anatomical compromise of the cervix such that the cervical reservoir for sperm in the ewes is greatly reduced. Since sperm recovered from the cervices of clover-affected ewes exhibit decreased motility (17), it appears that the phytoestrogen effect makes the mucus relatively "hostile" in the classic sense of cervical factor infertility. Such spermatoxicity is not understood in general nor in this specific case.

At higher phytoestrogen dosage, both higher volume and water content of cervical mucus are

observed in ewes (17,18), thus indicating that both cervical glycoprotein production and water excretion in the mucus are affected.

The cervical effects of phytoestrogens likely depend upon estrogen receptor mediation. In ewes, phytoestrogen treatment increases the rate of protein and glycoprotein synthesis and the number of estrogen binding sites in the cervix, but binding affinity remains unchanged (19). This finding implies that exogenous estrogen not only occupies the available binding sites, but stimulates the local production of more sites. Such receptor "up-regulation" may make the tissue more sensitive to estrogen action, and, if estrogen exposure continues, the cervical alterations would become more exaggerated.

Uterus

Pronounced uterine effects of phytoestrogens are also observed. The most notable uterine change that occurs is a marked increase in its weight relative to body weight, which constitutes the classic bioassay for estrogen action. A dose-dependent uterine weight increase is precipitated by acute administration of an extract of the Indian herb *Achyranthes aspera* in rats and hamsters at contraceptive dosage (75 mg/kg) and with as little as 1/20 this dosage (20). Similar results have been observed in mice, rats, and hamsters with only 1/40 contraceptive dose of ferujol extract (21). Stob (4) suggests that this hypertrophy of the uterus is the result of "typical estrogenic mechanisms," implying estrogen-receptor mediation. However, a more complex response to daily s.c. injection of female lambs with the phytoestrogen β-sitosterol has been reported, in which uterine weight increases for the first two weeks of treatment but markedly decreases over the next six-week period (22). Plausible explanations for such biphasic results include receptor "down regulation" and induction of metabolic enzymes with enhanced clearance of β-sitosterol. Similar results were obtained using ovariectomized ewes as the model (23).

Another manifestation of the uterotropic effect of phytoestrogens is seen in ewes suffering from infertility due to prolonged exposure to these agents. A marked increase in activity of some uterine enzymes and uterine DNA, protein, and glycoprotein synthesis occurs in such sheep (19). This observation indicates that at least a portion of the uterine weight gain is true hypertrophy rather than simply edema. At the same time, lower levels of lipids within the uteri of sheep fed phytoestrogen suggest inhibition of synthesis or increased utilization of lipids within this organ (22). Thus phytoestrogens may be affecting different enzymes in different fashions, stimulating the activity of some while blocking the action of others. It is noteworthy that the uterine RNA-to-DNA ratio decrease that occurs following ovariectomy is smaller in clover-affected than in normal ewes. This response is accompanied by less regression of the uterus in clover affected ewes than in controls. These findings indicate that phytoestrogenic action may be mediated via differentiations similar to those induced by hormonal steroids during fetal development (24).

Gross structural lesions of the uterus may also result from phytoestrogen exposure and could account for some instances of permanent sterility. Lesser lesions entail the proliferation of cystic endometrium, myometrial fibrosis, and endometrial fibrosis (13). These lesions could certainly compromise normal implantation of the conceptus. The most severe structural failure, complete uterine prolapse, is known to occur in some species following ingestion of some dietary estrogens (mycoestrogens) and obviously disrupts the reproductive process.

It is not clear whether phytoestrogens play any role in pregnancy wastage, but some plant preparations have been used as abortifacients. *Achyranthes aspera*, a common Indian herb claimed to possess abortifacient activity, did induce abortion in mice and rabbits, but failed to show similar effects in rats (20). It is uncertain whether a phytoestrogen is the active agent of *Achyranthes* that brings about abortion, but support for that possibility derives from the finding that miroestrol, a phytoestrogen from a legume tree root, is used by Burmese and Thai women in plant extract form to induce abortion (25). The mechanism for such an abortifacient action of these compounds is unstudied and any effects of phytoestrogens on uterine contractility *per se* have not been determined in either the gravid or nongravid state.

Phytoestrogen effects on uterine function may relate to alterations in activity of several enzymes. Under normal circumstances, oxidative enzymes in the uterus show slight reactions in the endometrium and uterine glands, but after administration of β-sitosterol, these weak reactions are curtailed (22). Such an inhibition of oxidative enzymatic activity in the uterine endometrium and glands may reduce local energy production due to an inability to replenish NAD+ and NADP+. This circumstance would diminish the ability of the uterus to contract and might decrease secretory capabilities of the uterine glands.

Alkaline phosphatase in the uterine tissue of ewes also responds to β-sitosterol in a biphasic pat-

tern. Alkaline phosphatase activity increases over the first two weeks of daily β-sitosterol injections and decreases over the second two weeks of injections (22). This disturbance in alkaline phosphatase activity may alter cell permeability and transport of nutrients by uterine cells.

Acid phosphatase activity in the uterus decreases with increasing dose and time of daily β-sitosterol treatments over an eight-week span (22). Such an inhibition would decrease free phosphorous, and may relate to the more general observation of decreased plasma phosphorus levels in exposed animals.

Uterine cholinesterase activity also decreases following β-sitosterol treatment, as evidenced by its diminished activity towards acetylthiocholine (22). This inhibition of activity is accompanied by a downward shift in sodium ion transport and decreased sodium in the uterine luminal fluid. It is not clear whether effects on sodium transport and cholinesterase activity are coincidental or truly associated processes in this instance.

Ovaries

While many anatomical effects of phytoestrogens have been described, physiologic changes in the reproductive tract are more subtle, but perhaps more consequential. Ovarian cyclicity may be disrupted by phytoestrogen exposure in mammals and birds (2,14,25,26), but interruption of ovulation due to short-term phytoestrogen ingestion is reversible (26). It is plausible that human vegetarians may have ovulatory dysfunction but suffer no other obvious physiologic abnormalities due to their diets (14). Abnormalities of ovulation may be due to direct ovarian actions since administration of β-sitosterol to ewes inhibited follicular development and altered the size distribution of follicles (22). Follicles were observed to show degeneration with intrafollicular hemorrhage and the development of shrivelled oocytes with lipid inclusions. The suggestion of a direct ovarian action of phytoestrogens in perturbing follicular maturation may be supported to some extent by a study which showed that in rats intraperitoneal administration of an extract from a plant species known to contain high concentrations of phytoestrogens inhibited follicular maturation (26). Obviously, these studies cannot distinguish between direct ovarian and indirect effects on follicular growth.

More direct evidence that the follicle may be a site of phytoestrogen activity derives from in vitro cultures of bovine granulosa cells. In this system, lower dosages of genistein and biochanin A increased progesterone synthesis while higher dosages inhibited progesterone synthesis (27). Since progesterone is essential in the establishment and maintenance of pregnancy, such an inhibition of progesterone production would be a plausible explanation for both failure of conception and early pregnancy wastage.

The possibility that phytoestrogens might be toxic to oocytes or early embryos was suggested in a single study (7). Mice fed coumestrol and then mated produced degenerate embryos exhibiting unevenly distributed cytoplasm and lack of symmetry in size among blastomeres, suggesting alterations in cleavage rates. Extensive vacuolization found in the ova also suggests that failure of fertilization of these ova may account for part of the observed decrease in litter size in mice fed coumestrol.

The activities of two ovarian enzymes appear to be influenced by phytoestrogens. First, low doses of phytoestrogen inhibit 17,20-lyase in bovine granulosa cells (27). This effect could profoundly alter the pattern and capacity of the steroidogenic pathways within the follicle or corpus luteum. The precise mechanism by which this effect occurs is unproven. Second, alkaline phosphatase in the ovaries is affected by phytoestrogen exposure (22). While the overall alkaline phosphatase activity is about equal in the ovaries of β-sitosterol-treated and control ewes, the control ewes show an intense reaction in the zona pellucida with a weak reaction in the interstitial tissue. Treated ewes exhibit an opposite response. Thus, a reversal of activities is seen where phytoestrogen is acting both to stimulate and to inhibit the same enzyme in two different sites within the ovary. While a mechanism for this action is not known, such changes in the activities of ovarian enzymes might compromise ovulation and increase the incidence of follicular degeneration in animals treated with phytoestrogens.

CNS/pituitary

Some phytoestrogen effects on ovarian function appear to result from indirect action on the secretion of gonadotropic hormones (7). In this context, there are four possible mechanisms of phytoestrogen action: 1) they are E_2 agonists, 2) they are E_2 antagonists, 3) they act as both E_2 agonists and antagonists, and 4) they act in a nonestrogenic capacity. Available information best supports the third of these possibilities (mixed agonist–antagonist effects). The site of phytoestrogen action could be the CNS (especially hypothalamus), the pituitary, or the gonad (see previous section).

The effect of intraperitoneal injection of phytoestrogen-rich *Dieffenbachia amoena* extract in rats on LH, follicle-stimulating hormone (FSH), prolactin (PRL), progesterone, and E_2 have been studied (26). In treated rats, levels of LH, FSH, and progesterone increased for doses of 2.5, 5.0, and 10.0 mg/kg of extract, while the levels of PRL and E_2 decreased at the same dosages. Progesterone levels showed a biphasic response, increasing at low doses of the extract (26), but decreasing at higher doses (27). Since no obvious single mechanism would explain all of these pituitary and ovarian hormonal changes, the extract may contain more than one endocrinologically active substance, or more than one site or mechanism of action might be involved.

There are data to suggest that phytoestrogens act both at CNS and pituitary levels to alter gonadotropin secretion. In both ovariectomized ewes (23) and intact clover-affected ewes (17), the best explanation for the impairment of gonadotropin secretion was a hypothalamic/CNS action. In particular, in clover-affected ewes, an LH surge could not be elicited by exogenous E_2 administration (consistent with loss of positive feedback), but the LH secretory response to exogenous gonadotropin-releasing hormone was normal (17), suggesting no pituitary effect. Our own data (28) show that acute phytoestrogen administration can alter GnRH-induced LH secretion in ovariectomized rats and thus suggest that the pituitary may be a site of phytoestrogen action in other situations.

Interactions between reproductive effects of phytoestrogen exposure and photoperiod in seasonal breeders have been investigated. In normal intact ewes, the frequency of LH pulses and plasma LH concentration are higher during breeding season than during anestrus season. In clover-diseased ewes, the frequency of LH pulses and LH concentration during breeding season are nearly the same as in normal ewes. In contrast during anestrus season, these LH pulse parameters remain at the high level of breeding season in clover-affected ewes, rather than decreasing as in normal ewes (18). These results suggest that a dissociation of normal photoperiod controls from the LH pulse generator may result from prolonged phytoestrogen exposure.

In ovariectomized ewes given estradiol implants, LH pulse frequency and amplitude vary seasonally, rather like the pattern seen in intact ewes. This seasonal variation in LH pulse frequency in ovariectomized ewes could depend upon extraovarian steroids from the adrenal glands, other intrinsic photoperiod-dependent CNS functional changes, or dietary estrogens. Results from one study suggest that dietary coumestrol decreases the amplitude of LH pulses but fails to affect the frequency of LH pulses or FSH concentrations during the breeding season (23). During anestrus, coumestrol does not alter any of these variables. Thus, coumestrol could only be partially responsible for the seasonal decrease in LH pulse frequency in ewes.

Sexual behavior

Changes in sexual behavior due to phytoestrogen exposure parallel the known physiologic effects. Clover-diseased ewes are slower than normal ewes to exhibit estrus behavior in response to either a single or several daily doses of E_2 (17,29,30). Accompanying the delayed estrus is a retarding of the first mount of the ewes by the ram, although the number of days on which the ewes allowed the ram to mount them does not significantly differ from controls. A delay of estrus in mice fed coumestrol also occurs (7), implying an antiestrogenic effect.

Apparent defeminization of the sexual behavior response following consumption of phytoestrogens is displayed by clover-affected ewes. These ewes show aggressive behavior, such as challenging and head bunting of rams and other ewes, sooner than control ewes following administration of testosterone (17). At the same time the ewes are slower in showing female behavior, such as standing to be mounted by a ram. Furthermore, clover-affected ewes exhibited less soliciting behavior than normals. However, the number of ewes that stood to be mounted decreased equally over the five-week period during which daily testosterone injections were given (30). Relative to controls, clover-diseased ewes exhibit a significantly greater degree of courting behavior 28 but not 21 days following treatment with testosterone. Other courting behaviors that are less hormonally dependent, such as anal and genital sniffing by the ewes, are not altered (17,30). While mechanisms for these behavioral effects are not known, we do know that females and males have similar numbers of estrogen binding sites in the hypothalamus, but estrogen-receptor complexes appear to have shorter nuclear acceptor occupancy in males than in females (31). Behavioral changes in clover-affected ewes could result from a change as simple as a decrease in nuclear acceptor occupancy by estrogen-receptor complexes.

E_2 causes a dose-dependent increase in the incidence and duration of hormone-dependent behaviors in ewes (Table 2), whereas E_2 has no effect on hormone-independent behaviors (30). The E_2-

Table 2. Estradiol-dependent and -independent behaviors in ewes

Hormone-dependent behaviors	Hormone-independent behaviors
Active soliciting	Squatting
Standing for mounting	Looking over shoulder
Allowing ram to mount	Tail fanning
	Kicking

induced behaviors occur less in phytoestrogen-affected ewes than in normals, while E_2 independent behaviors occur with equal frequency in control and clover-diseased ewes. Since general behavior appears normal but female sex-specific behavior is compromised in phytoestrogen-treated ewes, reproductive success could be compromised on a behavioral basis. The relationship of phytoestrogen-induced anatomic changes in the external genitalia and sexual behavior is not defined, but coital biomechanics could be altered as a result of such end organ effects. While vulvar and vaginal hypertrophy has been noted in various animals, masculinization has been observed in ewes (17) with clitoromegaly and fusion of the ventral commissure. Upon removal from estrogenic pasture, these changes do not reverse and could, therefore, permanently alter sexual function.

Phytoestrogenic effects in males appear to be consistent with expectations for exogenous administration of bioactive estrogen. Coumestrol increases teat length in wethers (23) and stimulates mammary hypertrophy in intact males. Rams grazed on estrogenic clover have reduced sperm counts (14), but it is not clear whether fertility is affected.

GENERAL METABOLIC EFFECTS IN MAMMALS

Protein synthesis

Some data suggest that phytoestrogens affect levels of plasma proteins. The effects of β-sitosterol on plasma concentrations of albumin, alpha-globulin, beta-globulin, gamma-globulin, and fibrinogen have been studied (32). Normal functions of these proteins are indicated in Table 3 (33). Even though total plasma protein concentration in mice is unaffected by s.c. administration of β-sitosterol, daily 25 to 100 μg injections of the agent increase four of the plasma proteins, but significantly decrease the gamma-globulin complex. The mechanisms of action of phytoestrogens in this system

Table 3. Plasma protein fractions affected by β-sitosterol*

Protein	Function	Effect of β-Sitosterol
Serum albumin	Regulation of blood volume; transport of fatty acids	Increase
Alpha-globulins	Transport of lipids, thyroxine, adrenal cortical hormones, and copper	Increase
Beta-globulins	Transport of lipids, iron, and hemes	Increase
Gamma-globulins	Act as most of the circulating antibodies	Decrease
Fibrinogen	Precursor to fibrin of blood clots	Increase

*(See reference 32).

are not established. It is likely that the phytoestrogens stimulate hepatic protein synthesis but inhibit production of gamma-globulins by lymphoid tissues. It is possible that the increased alpha-globulin concentration is a compensatory occurrence to erythrocyte count reduction that occurs following administration of β-sitosterol, thereby maintaining normal blood viscosity in the absence of normal erythrocyte concentration. The increase in the beta-globulin–fibrinogen complex appears to be correlated with its affinity for binding phosphorus. This affinity increases in response to β-sitosterol (32).

Enzyme activity of the liver

Phytoestrogens influence enzymes in nonreproductive as well as reproductive tissues. A relation between diet and synthesis of three enzymes in the liver of cheetahs has been shown. The affected enzymes, alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyltransferase, decrease in amount when cheetahs are taken off a diet high in soya bean content (thus high in phytoestrogen content) and given a chicken diet (13).

Inorganic plasma constituents

Phytoestrogens induce mineral changes in the blood. Subcutaneous injections of 25, 50, 75, or 100 μg of β-sitosterol increase calcium levels in mice, while doses of 5 or 10 μg of the phytoestrogen have no effect on calcium levels (34). Since E_2 inhibits bone mobilization, β-sitosterol may act by causing a decrease in E_2 levels via inhibition of gonadotropin secretion from the pituitary. Decreased ovarian E_2

production might then result in increased bone mobilization and increased serum calcium. Surprisingly, blood plasma phosphorus levels decrease following administration of 5 to 75 μg doses of β-sitosterol in mice, but show little change in response to a 100 μg dose (34). Decreases in phosphorus could be due to an enhanced rate of storage in an extravascular compartment, increased utilization of phosphorus by tissues, or increased renal clearance.

While β-sitosterol doses of less than 5 μg fail to change plasma magnesium levels, higher doses decrease plasma magnesium and increase both hepatic and intramuscular magnesium (34). Since magnesium is a smooth muscle relaxant, changes in uterine or tubal smooth muscle motility could result indirectly from this phytoestrogen action.

PHYTOESTROGENS IN HUMAN DISEASE

Deleterious roles

Phytoestrogens have been suggested to play both deleterious and beneficial roles with regard to illness. In the diets of cheetahs, phytoestrogens cause vascular hepatic lesions, in which the centrilobular and sublobular hepatic veins are partially or totally occluded (13). The possibility of human hepatic dysfunction must therefore at least be considered.

Vascular disease may be correlated with the consumption of dietary phytoestrogens (35). Coronary heart disease has been suggested to be associated with phytoestrogens consumed indirectly through the milk of cows; that is, the lactating cow consumes the phytoestrogens while grazing and, in turn, phytoestrogens in cow's milk are consumed by humans. One basis for this proposal is that phytoestrogens have more structural similarity to DES, a potent synthetic estrogen found to have atherogenic properties, than to endogenous estrogens such as E_2. The higher rate of coronary heart disease in human males might be explicable in part if human females are found to be better able to metabolize and excrete phytoestrogens.

Dietary estrogens could be a factor in cancer initiation in hormone responsive tissues, but no such instances have been demonstrated. Certainly phytoestrogens bind to both rat and human mammary tumor tissue and show competitive binding for mammary tissue E_2 receptors (15) raising the possibility of stimulation of estrogen-dependent neoplasms.

Beneficial roles

Estrogens have two opposing effects on cancer, depending on dosage. Large doses inhibit breast cancer tumor development and suppress growth of tumors already present, but small doses seem to promote tumor development and stimulate growth (36). This duality extends to phytoestrogens. Phytoestrogens may stimulate or inhibit tumor growth (8,14). One mechanism by which phytoestrogens may manifest their antitumor effects is blockade of estrogen receptors and uncoupling of receptor-mediated response. Thus the ability of endogenous estrogens to support tumor growth would be reduced. Indirect demographic support for a phytoestrogen-mediated reduction in cancers of hormone-responsive tissues might derive from the observation that women in countries consuming vegetarian diets have a lower incidence of breast cancer than in societies where a meat and vegetable diet is consumed (37).

Phytoestrogens may have antiviral and fungicidal properties (37), but a mechanism is not known. Support for the notion that this group of compounds could have such properties may lie in noting that the antifungal drug, ketaconazole, is also a potent inhibitor of some steroidal enzymes.

Plant estrogens have been implicated in the reduction of serum cholesterol levels in humans and animals with hypercholesterolemia. Such action is likely related to the role estrogens play in the metabolism and interaction of lipoproteins with regulation of cholesterol (8).

A final beneficial phytoestrogenic effect is alleviation of vasomotor symptoms in menopausal women. Historically the Chinese have used herbal medicine to treat "hot flushes." These herbal medications work as well as Premarin (an equine conjugated estrogen) in the mitigation of these symptoms in women with natural menopause (38). Similarly, the mycoestrogen, zearalanol, has been reported to reduce the incidence of hot flushes in women with surgical menopause (4). These effects would be consistent with the expected estrogenic properties of these compounds.

CONCLUSION

Phytoestrogens influence mammalian reproductive processes and can thereby compromise the reproductive success of individual mammals and possibly function as a selective environmental factor for populations. While phytoestrogens have a few propitious effects, the majority of the effects are nocuous. These compounds act through their similarity to endogenous estrogens and compete with the endogenous estrogens for binding sites.

Short-term effects of phytoestrogens seem to result from their mixed agonist-antagonist effects on estrogen-mediated processes in mammals. Since long-term exposures can produce persistent, even permanent anatomic, physiologic, or behavioral changes, phytoestrogens must affect the differentiation of some reproductive tissues and irreversibly alter the integration of mammalian reproductive processes in susceptable species.

REFERENCES

1. Schinckel PG. Infertility in ewes grazing subterranean clover pastures. Observations on breeding behavior following transfer to "sound" country. Austr Vet J. 1948;24:289–294.
2. Leopold AS, Erwin M, Oh J, Browning B. Phytoestrogens: adverse effects on reproduction in California quail. Science. 1976;191:98–99.
3. Fowler ME. Plant poisoning in free living wild animals: a review. J Wildlife Dis. 1983;19:34–43.
4. Stob M. Naturally occuring food toxicants: estrogens. In: Rechnig M Jr, ed. Handbook of naturally occurring food toxicants. Boca Raton: CRC Press; 1983:81–100.
5. Ollis WD. The isoflavonoids. In: Geissman TA, ed. *The chemistry of flavonoid compounds*. Los Angeles: Pergamon Press; 1962:353–405.
6. Hughes CL Jr. Phytochemical mimicry of reproductive hormones and modulation of herbivore fertility by phytoestrogens. Environ Health Perspect. 1988;78:171–175.
7. Fredricks GR, Kincaid RL, Bondioli KR, Wright RW. Ovulation rates and embryo degeneracy in female mice fed the phytoestrogen, coumestrol. Proc Soc Exp Biol Med. 1981;167:237–241.
8. Setchell KDR. Naturally occurring non-steroidal estrogens of dietary origin. In: McLachlan JA, ed. *Estrogens in the environment*. New York: Elsevier Press; 1985:69–85.
9. Mendelson JH, Mello NK, Ellingboe J, Skupny AST, Lex BW. Marijuana smoking suppresses luteinizing hormone in women. J Pharmacol Exp Ther. 1986;237:862–866.
10. Asch RH, Fernandez EO, Smith CG, Pauerstein CJ. Blockage of the ovulatory reflex in the rabbit with delta-9-tetrahydrocannabinol. Fertil Steril. 1979;31:331–334.
11. Sauer MA, Rifka SM, Hawks RL, Cutler GB, Loriaux DL. Marijuana: interaction with the estrogen receptor. J Pharmacol Exp Ther. 1983;224:404–407.
12. Kitts DD. Studies on the estrogenic activity of a coffee extract. J Toxicol Environ Health. 1987;20:37–49.
13. Setchell KDR, Gosselin SJ, Welsh MB, Johnston JO, Balistreri WF, Kramer LW, Dresser BL, Tarr MJ. Dietary estrogens — a probable cause of infertility and liver disease in captive cheetahs. Gastroenterology. 1987;93:225–233.
14. Setchell KDR, Borriello SP, Hulme P, Kirk DN, Axelson M. Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. Am J Clin Nutr. 1984;40:569–578.
15. Verdeal K, Brown RR, Richardson T, Ryan DS. Affinity of phytoestrogens for estradiol binding proteins and effect of coumestrol on growth of 7,12-dimethylbenz[a]anthracene-induced rat mammary tumors. J Nat Cancer Inst. 1980;64:285–290.
16. Li JJ, Li SA, Klicka JK, Heller JA. Some biological and toxicological studies of various estrogen mycotoxins and phytoestrogens. In: McLachlan JA, ed. Estrogens in the environment. New York: Elsevier Press; 1985:168–183.
17. Adams NR. A changed responsiveness to estrogen in ewes with clover disease. J Reprod Fertil. 1981;30(Supplement): 223–230.
18. Chamley WA, Clarke IJ, Moran AR. Seasonal changes in LH secretion in normal ewes and ewes which grazed oestrogenic clover. Austr J Biol Sci. 1985;38:109–113.
19. Tang BY, Adams NR. Oestrogen receptors and metabolic activity in the genital tract after ovariectomy of ewes with permanent infertility caused by exposure to phytoestrogens. J Endocrinol. 1981;89:365–370.
20. Wadhwa V, Singh MM, Gupta DN, Singh C, Kamboj VP. Contraceptive and hormonal properties of *Achyranthes aspera* in rats and hamsters. Planta Medica. 1986;52:231–232.
21. Singh MM, Gupta DN, Wadhwa V, Jain GK, Khanna NM, Kamboj VK. Contraceptive efficacy and hormonal profile of ferujol: a new coumarin from *Ferula jaeschkeana*. Planta Medica. 1985;51:268–270.
22. El Samannoudy FA, Sjareja AM, Ghannudi SA, Gillaly GA, El Mougy SA. Adverse effects of phytoestrogens: effect of β-sitosterol treatment on follicular development, ovarian structure, and uterus in the immature female sheep. Cell Molec Biol. 1980;26:255–266.
23. Montgomery GW, Martin GB, Le Bars J, Pelletier J. Gonadotrophin release in ovariectomized ewes fed different amounts of coumestrol. J Reprod Fertil. 1985;73:457–463.
24. Tang BY, Adams NR. Properties of nucleic acids in the uteri of ewes with clover disease and the effect of oestrogen after ovariectomy. Austr J Biol Sci. 1982;35:527–531.
25. Harborne JB. Introduction to ecological biochemistry. 2nd ed. New York: Academic Press; 1982:100–106.
26. De Pasquale RC, Ragusa S, Circosta C, Forestieri AM. Investigations on *Dieffenbachia amoena gentil*. Endocrine effects and contraceptive activity. J Ethnopharmacol. 1984;12:293–303.
27. Kaplanski O, Shemesh M, Berman A. Effects of phytoestrogens on progesterone synthesis by isolated bovine granulosa cells. J Endocrinol. 1981;89:343–348.
28. Hughes CL Jr. Effect of phytoestrogens on GnRH-induced luteinizing hormone secretion in ovariectomized rats. Reprod Toxicol. 1988;1:179–181.
29. Adams NR. Sexual behaviour responses of the ovariectomized ewe to oestradiol benzoate, and their persistent reduction after exposure to phyto-oestrogens. J Reprod Fertil. 1978;53:203–208.
30. Adams NR. Sexual behaviour of ewes with clover disease treated repeatedly with oestradiol benzoate or testosterone propionate after ovariectomy. J Reprod Fertil. 1983;68: 113–117.
31. Barley J, Ginsburg M, MacLusky NJ, Morris ID, Tomas PJ. Sex differences in the distribution of cytoplasmic oestrogen receptors in rat brain and pituitary effects of gonadectomy and neonatal androgen treatment. Brain Res. 1977;129:309–318.
32. Hassanein RR, Elmougy SA, Elghamry MI. Biological activity of phytoestrogens: fractionation of plasma proteins associated with β-sitosterol treatment. Planta Medica. 1972;22:412–417.
33. Lehninger AL. *Principles of biochemistry*. New York: Worth Publishers; 1982:707.
34. Elghamry MI, Hassanien RR, Elmougy SA. Mineral changes in the blood of ovariectomized mice after treatment with β-sitosterol. Zeitschrift fur Klinische Chemie un Klinische Biochemie. 1971;9:346–347.
35. Seely S. The possible connection between phytoestrogens, milk, and coronary heart disease. Med Hypoth. 1982;8:349–354.
36. Martin PM, Horwitz KB, Ryan DS, McGuire W. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology. 1978;35:1860–1867.
37. Adlercreutz H, Fotsis T, Bannwart C, Wahala K, Makela T, Brunow G, Hase T. Determination of urinary lignans and phytoestrogen metabolites, potential antiestrogens and anticarcinogens, in urine of women on various habitual diets. Steroid Biochem. 1986;25:791–797.
38. Mochimaru F, Toyama M, Kanakura Y, Inde S. Objective indicator for the assessment of postmenopausal hot flashes. Acta Obstet Gynaecol Jpn. 1984;36:643–645.

evoked oto-acoustic emissions. *Arch Otolaryngol Head Neck Surg* 1988;114: 887-90.
9 Jacobson JT, Morehouse CR. A comparison of auditory brain stem response and behavioural screening in high risk and normal newborn infants. *Ear Hear* 1984;5:247-53.
10 Mahoney TM, Eichwald JG. The ups and "downs" of high risk screening: the Utah statewide program. *Seminars in Hearing* 1987;8:155-63.
11 Stein L, Clark S, Kraus N. The hearing impaired infant: patterns of identification and habilitation. *Ear Hear* 1983;4:232-6.
12 Elssmann SF, Matkin ND, Sabo MP. Early identification of sensorineural hearing impairment. *The Hearing Journal* 1987;40:13-7.
13 American Speech-Language-Hearing Association. Guidelines for the identification of hearing impairment in at risk infants age birth to 6 months. *ASHA* 1988;30:61-4.
14 Hendricks-Munoz KD, Walton JP. Hearing loss in infants with persistent fetal circulation. *Pediatrics* 1988;81:650-6.
15 Nauby CM, Weiss IP, Hetzer GR. Progressive sensorineural hearing loss in survivors of persistent fetal circulation. *Ear Hear* 1986;7:74-7.
16 Hall DMB, ed. Screening for hearing impairment. In: *Health for all children*. Oxford: Oxford Medical Publications, 1989:54-61.

(Accepted 24 August 1990)

Catheterisation: your urethra in their hands

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Br Med J 1990;301:905

The emphasis in undergraduate medical education is often on the theoretical aspects of medicine rather than the practical aspects. Practical procedures are commonly taught informally, the teaching being passed from one junior to the next.[1] The philosophy is of "See one, do one, teach one." Urethral catheterisation is a procedure that requires a certain amount of skill, knowledge, and experience and is not without complication,[2-4] yet it is usually delegated to the most junior and inexperienced medical staff, the junior house officers.

Subjects, methods, and results

To assess the level of competence at catheterisation among junior medical staff house officers at this hospital were interviewed with a structured questionnaire, covering three aspects of the procedure: the degree of undergraduate and postgraduate instruction, the practical and theoretical aspects of catheterisation, and, finally, problems and complications encountered.

Thirty junior house officers (graduates of five medical schools) were interviewed. Eighteen were male and 12 were female. The replies to the questionnaire showed that none of the interviewees had received any formal instruction regarding any aspect of urethral catheterisation as an undergraduate. Practical postgraduate instruction in 24 was limited to supervision of a single catheterisation, and four subjects were unsupervised. Although those interviewed had performed a mean of 28 (range 6-100) catheterisations in male patients, only four of them had catheterised female patients.

Despite the large number of procedures performed there was appreciable ignorance of the practical and theoretical aspects of catheterisation. Twenty five interviewees were unaware of the availability of short term and long term catheters or of the duration for which they may be safely left without being changed. Three interviewees simply used the catheter that was provided by the nursing staff, and one did not know that different sizes existed.

Twenty eight interviewees initially used force when meeting resistance to the passage of the catheter, and 13 stated that the development of fresh urethral bleeding would not deter them from a further attempt at catheterisation. Eighteen were happy to attempt catheterisation in a patient who had a known urethral stricture. Five interviewees were unaware of the difference between a phimosis and paraphimosis.

Despite the lack of formal tuition all had developed what seemed to be a satisfactory aseptic technique. None, however, was aware of the nature of the antiseptic fluid or the strength of the local anaesthetic gel, but simply used what was provided by the nursing staff.

Nineteen of the interviewees had encountered bleeding and six had had patients in whom a paraphimosis had developed after catheterisation. A particularly disturbing finding was that, although 14 interviewees had requested help from senior medical staff, seven were reluctant to seek advice, because of their impression that difficulties with catheterisation were not worthy of disturbing senior staff. Eight of the 12 female medical staff had encountered problems with male patients becoming sexually excited during the procedure.

Discussion

The results of our survey suggest that the technique of urethral catheterisation is poorly taught, and in the light of these results we are preparing a short teaching video to be shown to every house officer at the start of their preregistration post.

1 Royal College of Physicians. *Resuscitation from cardiopulmonary arrest. Training and organisation.* London: RCP, 1987.
2 Blandy JP. Emergency situations. Acute retention of urine. *Br J Hosp Med* 1987;19:109-11.
3 Merguerian PA, Erturk E, Hulbert WC, et al. Peritonitis and abdominal free air due to intraperitoneal bladder perforation associated with indwelling urethral catheter drainage. *J Urol* 1985;134:747-50.
4 Macfarlane DE. Prevention and treatment of catheter-associated urinary tract infections. *J Infect* 1985;10:96-106.

(Accepted 8 August 1990)

Oestrogenic effects of plant foods in postmenopausal women

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Br Med J 1990;301:905-6

Crops grown as animal pasture are known to have oestrogenic activity,[1] and some foods contain potential oestrogenic analogues such as the isoflavonoids (isoflavones and coumestans), lignans, and resorcyclic acid lactones,[2] which may be activated or inactivated.[3] We studied the effect of three foods reported to induce vaginal oestrus in laboratory animals[4] in postmenopausal women not taking oestrogen replacement therapy.

Subjects, methods, and results

We studied 25 postmenopausal women who were non-smokers, in good general health, and taking no drugs known to affect oestrogen state (mean age 59 (range 51-70); body mass index 24·4 (range 18·7-31·6) kg/m²; years after menopause 8·1 (range 1-20)). The protocol was a latin square design with a two week run in period and a six week experimental period. The women recorded their normal diet for 14 days and were asked to repeat the fortnightly diet throughout the study. During the experimental period the diet was

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supplemented with soya flour (45 g daily), red clover sprouts (10 g dry seed daily), and linseed (25 g daily), each for two weeks in turn. To check compliance the women returned residual food. Blood samples were taken weekly and lateral wall vaginal smears taken fortnightly and at follow up two and eight weeks after supplementation finished. Analysis was on intention to treat, but 23 women completed the study.

We examined the dependent variables vaginal cell maturation and serum concentrations of luteinising hormone and follicle stimulating hormone. The cumulative effects of the three foods at six weeks were compared with baseline by the paired *t* test, as were the residual effects, two and eight weeks after the last food supplement. We found significant differences in vaginal cytology after six weeks' supplementation ($p<0.01$, 95% confidence interval 6·0 to 17·6), which persisted for two weeks after treatment ($p<0.02$), but cytology returned to baseline after eight weeks (table).

Mean (SE) values for oestrogenic indicators in postmenopausal women consuming phyto-oestrogens

Week		Maturation value	Luteinising hormone (IU/l)	Follicle stimulating hormone (IU/l)
1			45·7 (3·1)	58·7 (2·7)
2		30·8 (4·5)	46·6 (3·4)	58·7 (3·0)
3			50·8 (8·5)	57·6 (2·2)
4		35·0 (5·1)	46·0 (3·6)	57·3 (2·9)
5	Food		46·2 (3·1)	57·7 (3·0)
6	supplementation	39·6 (5·3)	42·9 (3·2)	54·3 (2·9)
7			42·6 (3·3)	56·4 (2·8)
8		43·4 (3·6)	44·6 (3·3)	56·6 (2·4)
9			44·9 (3·5)	57·9 (2·8)
10		43·6 (4·7)	44·9 (3·5)	57·5 (2·7)
16		33·7 (5·5)		

The maturation value significantly increased after soya flour ($p<0.05$) and linseed ($p<0.02$) but not after red clover sprouts ($p=0.11$).

All women had concentrations of follicle stimulating hormone and luteinising hormone greater than those in the premenopausal range of 2·8 IU/l and 6·13 IU/l respectively. There was a cumulative effect on serum concentrations of follicle stimulating hormone ($p<0.05$) but not on luteinising hormone over the six week supplementation period. Individual two week food supplements had no measurable effects on either hormone.

In seven women with the most pronounced changes in vaginal cytology we measured serum oestradiol concentrations weekly. Baseline concentrations were <70 pmol/l in all but one woman, who was retained as the study was based on intention to treat. There were no appreciable changes in body weight during the study.

Comment

We aimed to consider whether phyto-oestrogens were of consequence in human nutrition. Our study gives some indication of the recovery time from any possible effect of treatment and also provides further evidence of causality. Vaginal maturation is a sensitive and specific indicator of oestrogenicity. Follicle stimulating hormone is less sensitive to weak oestrogenic compounds such as phyto-oestrogens. Weak oestrogenic compounds may sometimes act as anti-oestrogens, which may affect their usefulness as sources of oestrogenic activity. Conversely, tamoxifen, an antioestrogen, can have oestrogenic effects on vaginal cytology.[5]

Patterns of food intake may modulate the severity of the menopause as it is an oestrogen deficiency state. Up to half of the diet of some populations may comprise foods containing phyto-oestrogens, whereas in our study such foods comprised only about 10% of energy intake for a fairly short time. Whether menopausal symptoms differ in such populations would be worth investigation.

We thank our statistical adviser, Steve Forrish, from the department of social and preventive medicine, Monash University.

1 Schutt DA. The effects of plant oestrogens on animal reproduction. *Endeavour* 1976;35:110–4.
2 Price KR, Fenwick GR. Naturally occurring oestrogens in foods—a review. *Food Addit Contam* 1985;2:73–106.
3 Adlercreutz H, Fotsis T, Bannwart C, *et al.* Determination of urinary lignans and phytoestrogen metabolites, potential antioestrogens and anticarcinogens, in urine of women on various habitual diets. *J Steroid Biochem* 1986;25:791.
4 Farnsworth NR, Bingel AS, Cordell GA, Crane FA, Fong HHS. Potential value of plants as sources of new antifertility agents. *J Pharm Sci* 1975;64:717–54.
5 Vermeij E, Carol O, Matamoro R, Trevedas M. Oestrogen-like effect of tamoxifen on the vaginal epithelium. *Ann Med* 1977;3:135–2.

(*Accepted 19 June 1990*)

Inadvertent duplicate publication

Loop diathermy excision of the cervical transformation zone in patients with abnormal cervical smears

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The *BMJ* regrets that much of the material in the above article (30 June 1990, *p* 1690) was substantially the same as that published previously in *Contemporary Reviews in Obstetrics and Gynaecology* (Redman CWB, Buxton EJ, Cullimore J, Luesley DM. Loop diathermy excision of the cervical transformation zone in the management of cervical intraepithelial neoplasia. 1990;2:53–8). The authors did not tell us this when the article was submitted, their article did not contain any reference to the earlier paper, and all authors signed our copyright form, which states, among other things, that "papers are accepted on condition that they have not been published by any other journal."

We regret this inadvertent duplicate publication, for which the authors hold sole responsibility, and which is in violation of our Instructions to Authors and internationally agreed guidelines.

Correction

Incidence of peptic ulcer disease in Gothenburg, 1985

An editorial error occurred in this paper by Dr Ivy-Mai Schöön and others (1989;299:1132). The y axis of figure 1 should read 0, 5, 10, 15, and 20 and not 0, 0·5, 1·0, 1·5, and 2·0 as published.

D9

COMMENTARY

The Role of Soy Products in Reducing Risk of Cancer[1]

Mark Messina, Stephen Barnes*

Since the initial recognition that diet plays a role in the etiology of certain cancers, particularly cancers of the breast and colon, considerable progress has been made in identifying dietary patterns associated with cancer risk. There is general agreement that a high-fat, low-fiber diet, like that consumed by much of the industrialized world, increases cancer risk and that plant-based diets, rich in whole grains, legumes, and fruits and vegetables, are protective. It has been, however, considerably more difficult to identify specific foods, types of food, or components of foods that influence cancer risk.

The recent workshop on The Role of Soy Products in Cancer Prevention, sponsored by the National Cancer Institute, had two objectives: 1) to evaluate the role of soybeans, food products derived from soybeans, and specific components of soybeans in the dietary prevention of cancer and 2) to recommend research initiatives and approaches for further studies of the effect of soy intake on human cancer risk. The meeting was chaired by Stephen Barnes and organized by Mark Messina.

Isoflavones in Cancer Prevention

Kenneth Setchell, Donna Baird, and Barnes discussed the potential role of isoflavones in the prevention of cancer. Setchell reviewed the history of phytoestrogens (*1*), noting that plants were first observed to induce estrus in animals in 1926. Over 300 plants are now known to possess estrogenic activity (*2,3*). In 1946, the infertility observed in Australian sheep that grazed on a certain type of subterranean clover was attributed to the high isoflavone content of this plant (*4*). Ruminal bacteria in these animals convert plant isoflavones into the mammalian isoflavone equol, which, following absorption, may suppress the pituitary gonadotropic axis. Equol, a weak estrogen possessing about 0.2% of the biological activity of estradiol, was first identified in human urine in 1982 by Setchell et al (*5,6*). Setchell's further interest in the potent estrogenic effects of soybean isoflavones was stimulated coincidentally. He discovered that the soy component of diets fed to captive cheetahs, which was added for economic reasons, was responsible for the severe breeding problems in these animals (*6,7*).

Setchell noted that isoflavone metabolism has been studied in humans, although only superficially. In one study, subjects fed 40 g of soy daily were found to have urinary levels of equol as much as 1000-fold higher than baseline values (*8,9*). The low levels of urinary equol in two of the six subjects in this study indicate that the intestinal microflora (*10*) participate in isoflavone metabolism and that isoflavones undergo enterohepatic circulation (*10*). Improved analytical methods (*11,12*) have led to the realization that equol represents only a small fraction of the total amount of isoflavone in urine and that conjugates of the soybean isoflavones daidzein and genistein are the major forms present. The high levels of isoflavone in urine in subjects fed soy suggest that these compounds are likely to elicit a biological response (*13*).

Setchell concluded his presentation with a reminder (*a*) that all weak estrogens can also have antiestrogenic activity; (*b*) that tamoxifen, which has been used therapeutically for breast cancer, is structurally related to some of the phytoestrogens; and (*c*) that vegetarians, who may have a lower risk of certain cancers, excrete higher levels of phytoestrogens. These findings have led to collaborative studies by Barnes, Setchell, and associates (*14*), who used an animal model designed to test the hypothesis that phytoestrogens have a role in reduction of breast cancer risk.

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[1]Report of a workshop held June 26-27, 1990, at the Guest Quarters Hotel in Bethesda, Md. Workshop members were Donna Baird, National Institute of Environmental Health Sciences, Research Triangle Park, NC; Stephen Barnes, University of Alabama at Birmingham, Birmingham, Ala; David L. Brandon, Western Regional Research Center, United States Department of Agriculture, Albany, Calif; James A. Duke, Agricultural Research Service, United States Department of Agriculture, Beltsville, Md; Ernst Graf, The Pillsbury Co, Minneapolis, Minn; Ann R. Kennedy, University of Pennsylvania Medical School, Philadelphia; Renee M. Kosslak, Iowa State University, Ames; Irvin E. Liener, University of Minnesota, St. Paul; Mark Messina, National Cancer Institute, Bethesda, Md; Frank L. Meyskens, University of California, Irvine, Calif; A. Venket Rao, University of Toronto, Ontario, Canada; Kenneth D. R. Setchell, Children's Hospital, Cincinnati, Ohio; Bernie F. Szuhaj, Central Soya, Fort Wayne, Ind.

Received October 22, 1990; revised January 8, 1991; accepted January 16, 1991.

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Barnes began by observing that Oriental women, who have low incidence rates of breast cancer (*15*), consume larger amounts of soy products than do most American women. However, although fertility and reproduction in animals are adversely affected by ingestion of plant isoflavones, the amount of isoflavones in soy products consumed by Oriental women does not appear to affect their reproductive capacity.

Barnes discussed the recent animal study conducted in collaboration with Setchell and other investigators (*14*). In that study, consumption of soybeans significantly decreased chemically induced rodent mammary cancer. Rats were fed one of four soy products: powdered soybean chips consisting of unpurified soybeans, both raw and autoclaved; soy protein isolate composed of 91% protein; soy molasses, a concentrate of the aqueous alcohol extract of soy flour; and aqueous alcohol-extracted soy protein concentrate. All diets were isocaloric and isonitrogenous and produced similar weight gain among the animal groups throughout the study.

The first three products, all of which are rich in isoflavones, inhibited mammary tumorigenesis induced by 7,12-dimethylbenz[*a*]anthracene or methylnitrosourea, while the aqueous alcohol-extracted soy protein concentrate, which had a low content of isoflavones, did not. Whether the soybeans were raw or cooked made no difference in the degree of inhibition of mammary cancer; cooked soybeans were shown to be devoid of protease inhibitor activity.

Barnes said the reduction in levels of mammary tumor estrogen receptors induced by the powdered soybean chips paralleled the inhibition of tumorigenesis and supported the hypothesis that the isoflavones exerted an antiestrogenic effect. Interestingly, however, this was not the case for the soy protein isolate. The decrease in levels of mammary tumor estrogen receptors was smaller than predicted from the degree of tumor inhibition, he said, suggesting that the antiestrogenic effect of isoflavones may not be the primary mechanism responsible for inhibition of tumorigenesis. Therefore, Barnes concluded, the anticarcinogenic activity of isoflavones may not be limited to tumors containing a functional steroid receptor system. Alternative mechanisms of action may include inhibition of the activity of tyrosine protein kinases (eg, epidermal growth factor receptor tyrosine kinase) (*16*), DNA topoisomerase II (*17*), and ribosomal S6 kinase (*18*), as well as induction of specific cytochrome P450s (*19*).

Baird, before describing her recent study of the effects of feeding soy to postmenopausal women (manuscript in preparation), cited the concern of the National Institute of Environmental Health Sciences about the possible effects of low-level environmental estrogens on health. In her study, changes in estrogenic activity in postmenopausal women consuming soy over a 4-week period were examined. Soy was chosen for this study because of its high estrogenic activity (*20,21*), its increasing use in the United States, and the variety of products derived from soy and because soy consumption would not adversely affect nutritional status (*22*). Subjects consumed daily one main soy dish (½ cup of soybeans or 38 g of texturized vegetable protein) and two soy snacks—either soy chips (a roasted soybean product) or a spread for crackers made from the whole soybean. The estimated isoflavone content was about 200 mg/day, the equivalent of about 0.3 mg/day of conjugated steroidal estrogen, assuming that the estrogenic activity of phytoestrogens is about 0.1% that of conjugated estrogen.

Baird said preliminary findings indicate that, compared with control subjects, significantly more women fed soy exhibited an estrogenic response, as demonstrated by an increase in the number of superficial cells of the vaginal epithelium. She remarked that postmenopausal women were chosen for this study because of the decision to examine the estrogenic rather than the antiestrogenic effects of plant phytoestrogens. In premenopausal women with relatively high estrogen levels, the antiestrogenic effects of soybeans may have been observed.

Protease Inhibitors

Ann Kennedy, David Brandon, and Irvin Liener focused their attention on the soybean protease inhibitors. Kennedy reviewed her work, as well as that of others, in the field of protease inhibitors and cancer prevention. She noted that the soybean-derived Bowman-Birk protease inhibitor (BBI) either inhibits or prevents development of experimentally induced colon (*23*), oral (*24*), lung (*25*), liver (*23*), and esophageal cancers (von Hofe E, Newberne P, Kennedy A: unpublished observations). Protease inhibitors, at the levels used in these studies, do not adversely affect animal growth. Kennedy noted that the anticarcinogenic effect of the BBI is thought to stem from its ability to inhibit chymotrypsin activity (*26*), but results also suggest an important role for trypsin inhibition in suppression of the promotional stage of carcinogenesis (*27*). She said in vitro work indicates that protease inhibitors prevent conversion of normal cells to the malignant state even at very late stages in carcinogenesis but that they have no effect on cancerous cells (*28*). Protease inhibitors are unique in that they cause an irreversible suppressive effect on the carcinogenic process. They have also been shown to suppress oncogene expression and to inhibit carcinogen-induced protease activity (*29*).

Kennedy said recent data suggest that the antigrowth effects of raw soybeans commonly attributed to protease inhibitors may actually be due to an unidentified factor(s) (*30*). Furthermore, in human populations consuming soybeans, the connection between pancreatic enlargement and protease inhibitors observed in animals has not been seen. In fact, incidence of pancreatic cancer is decreased in these groups (*31*). Kennedy noted that in vitro comparisons of the pure BBI with an extract of soybeans containing BBI indicate that the activity of the soybean extract could be directly attributable to BBI (*26*). However, she said an in vivo study suggests that the extract may contain an additional anticarcinogenic agent working in conjunction with the BBI (*26*). The extract contains approximately 50% protease inhibitor; the remaining content is unknown, but it may include isoflavones as well as other potential anticarcinogens. Kennedy commented that the lowest effective dietary levels of protease inhibitors used in these animal studies (0.1%) could be achieved by humans by modifying the diet to include soy products.

Brandon discussed the measurement of protease inhibitors in soybeans and soy products, noting the concern of the Agricultural Research Service of the United States Department of Agriculture (USDA) over the possible adverse effects of

protease inhibitor intake in humans, particularly in infants (*32*). Enzyme-linked immunosorbent assays (ELISA), using monoclonal antibodies, have been developed for the measurement of two different protease inhibitors found in soybeans—BBI and Kunitz trypsin inhibitor (KTI) (*33,34*). These procedures are suitable for quantifying residual protease inhibitor levels in foods. A variety of processed soy products, a series of soybean flours derived from seeds in the USDA Soybean Germplasm Collection, and the soybean isoline L81-4590 (lacking KTI) (*35*) have been analyzed. Brandon noted that an important observation from the ELISA analysis of heat-treated soy flours derived from the isoline was that KTI, not BBI, is responsible for the heat-stable activity of commercial soy flour that inhibits trypsin activity (*36,37*). The microenvironment of the soy flour appears to promote heat inactivation of BBI to a greater extent than it affects KTI. This finding contrasts with the results of work showing that BBI is relatively heat stable in the pure form (*38*). Moisture, fat content, the presence of agents that influence changes in disulfide bonds, and interactions with other constituents, such as carbohydrates, appear to influence the denaturation of inhibitors (*39*).

Brandon said analysis of infant formula has revealed that active KTI and BBI, when measured on the basis of weight per gram of protein, are reduced to about 0.1% of their levels in raw soy (*40*). An infant on a diet consisting exclusively of soy formula would consume about 10 mg of active KTI plus BBI per day. In toasted (autoclaved) soy flour, 20%-30% of the KTI activity remains, while all of the BBI is inactivated. Analysis of tofu (soybean curd) has revealed that the protease inhibitor content varied significantly among the samples, from 4 to 30 μg of BBI and from 5 to 16 μg of KTI per gram of product. The protease inhibitor content of several soy protein isolates also varied, as much as 20-fold. Not unexpectedly, there was also a wide variation in the protease inhibitor content among varieties of soybeans. Brandon suggested that food-processing strategies could be combined with genetic approaches to optimize the protease inhibitor content of soy products.

Liener reviewed research on the potential adverse effects of consuming protease inhibitors, first noting that most work has been done with small experimental animals (*41*). Consumption of raw soybeans has two major effects: growth inhibition and pancreatic enlargement. Rats consuming raw soy flour for extended periods develop adenomatous nodules involving acinar cells of the pancreas (*42*). Additionally, raw soy flour consumption potentiates the effect of pancreatic carcinogens (*43*). In a study by Liener et al (*44*), heat treatment of raw soybeans almost completely eliminated this potentiation, while the addition of protease inhibitors to the heated product restored most of the pancreatic enlargement observed with raw soy, suggesting that protease inhibitors are at least partly responsible for pancreatic enlargement.

Liener noted that the varied response to raw soy flour among species is particularly important. Rats, mice, chickens, hamsters, and young, growing guinea pigs all exhibit pancreatic enlargement in response to protease inhibitors, while dogs, pigs, calves, and monkeys do not (*45*). Growth inhibition induced by soybean products is thought to result from a deficiency of the sulfur-containing amino acids caused by the dramatic increases in fecal levels of endogenous protease enzymes, particularly trypsin and chymotrypsin, two enzymes that are rich in these amino acids (*46*).

Commenting that pancreatic enlargement apparently stems from elevated serum levels of the hormone cholecystokinin, Liener commented that pancreatic enzyme secretion is inversely related to the level of trypsin in the intestine, a process regulated by cholecystokinin. This hormone stimulates the pancreas to produce trypsinogen, but because the protease inhibitors combine with trypsin, the suppressive effect of trypsin on intestinal release of cholecystokinin is eliminated (*47*).

Liener raised the question: Can the effects of protease inhibitors in small animals be extrapolated to humans? A negative feedback system in humans has been observed (*48*). Directly supplying BBI or raw soy flour to the duodenum causes an increase in secretion of pancreatic enzymes (*48*) and in blood levels of cholecystokinin (*49*). (BBI, in contrast to KTI, survives in gastric juice.) Despite these observations, he said, it is not possible at this time to accurately assess the health consequences of consuming processed soy products.

Phytosterols and Saponins

A. Venket Rao presented evidence for reduction of colon cancer risk by phytosterols and saponins. Both substances are common constituents of plants, but the concentration in soybeans is particularly high. Phytosterols are structurally similar to the animal sterol cholesterol. They inhibit cholesterol absorption and are almost quantitatively recoverable in fecal material, indicating that very little intestinal absorption occurs (*50*). Soybeans are a major contributor of phytosterols to the diet, particularly β-sitosterol (90 mg/100 g edible portion of the soybean) (*51*). Soybean oil is potentially an important source of phytosterols, but upon refinement and hydrogenation, phytosterol levels are reduced from 315 mg to 217 mg and 132 mg, respectively, per 100 g of oil (*51*). Dietary phytosterol intake among populations differs dramatically; the typical western diet contains about 80 mg/day, while Japanese and vegetarian diets provide about 400 and 345 mg/day, respectively (*52,53*).

In addition to the phytosterols, whole soybeans contain significant amounts of saponins, about 5% of dry weight (*54*), while tofu contains approximately half that much. Saponins are amphophilic compounds having surfactant properties and, like phytosterols, bind to cholesterol and bile acids.

Rao said that while nutritional interest in both phytosterols and saponins has focused on their cholesterol-lowering properties, some data suggest that these compounds may be anticarcinogens. In rats, β-sitosterol-supplemented diets (0.2% by weight) inhibit chemically induced colon cancer (*55*), and phytosterols reduce, in a dose-dependent fashion, cholic acid-induced colon cell proliferation and mitotic activity (*56*). Diets containing phytosterols at 1% by weight are well tolerated by experimental animals (*57*). Dietary saponins from soybeans and other sources have been shown to enhance immunity (*58,59*), are cytotoxic to Sarcoma 37 cells (*60*), inhibit DNA synthesis in tumor cells (*61*), decrease the growth of human epidermoid carcinoma cells (*62*) and human cervical carcinoma cells (*63*), and inhibit Epstein-Barr virus genome expression (*64*). Saponin-sup-

plemented diets (1% by weight), as is the case for the phytosterols, normalize abnormal colonic cell proliferative activity induced by carcinogens (Rao AV: unpublished observations).

Inositol Hexaphosphate

Ernst Graf discussed the rationale for the hypothesis in which inositol-1,2,3,4,5,6-hexaphosphate (IP_6), not fiber, is postulated to be responsible for the inverse correlation between the incidence of colon cancer and the consumption of fiber-rich foods (*65*). When the IP_6 content of cereals, fruits, and vegetables is considered, the international data suggest that there is a greater negative correlation between IP_6 and colon cancer incidence than between fiber and colon cancer incidence. IP_6 is found in a variety of plant foods, particularly cereals, but soybeans are an especially rich source, containing about 1.4% on a dry-weight basis (*66*).

Graf noted that most nutritional interest thus far has focused on the inhibitory effect of IP_6 on mineral absorption. IP_6 forms tight chelates with a variety of polyvalent metals such as calcium, zinc, and iron (*66*). However, he said, the ability to bind metal ions, particularly iron, may provide the basis for the anticarcinogenic effects of this compound. Graf commented that iron may be a key factor, via the Haber-Weiss reaction, in the production of hydroxyl radicals, which are postulated to play a role in the etiology of some cancers (*67*). IP_6 has been shown to limit the oxidant reactivity of transition metals (*66*), to inhibit lipid peroxidation (*67*), and to inhibit experimentally induced colon cancer (*68-73*). It has also been suggested that IP_6, through absorption following dephosphorylation to IP_3, could be an important second messenger involved in the regulation of cell differentiation (*73*).

Phytochemical Variation

James Duke discussed phytochemical variation in soybeans. Duke started by noting that there are over 10 000 named or numbered varieties of the common soybean *Glycine max L.* In these varieties, as one might expect, lies tremendous chemical variation. The genus *Glycine* was originally applied to a distant relative, now known as *Apios americana*, which is an edible root with more protein than is found in potato (*74*).

The isoflavone content of soybeans varies tremendously according to the plant part, variety, year harvested, and geographic location (*75*). Soybean hulls contain only relatively minor amounts of isoflavones, the majority of which occur in the hypocotyl, although one common isoflavone, genistein, is found primarily in the cotyledon (*75*). Equally significant are the reported differences in isoflavone content according to the varieties of soybeans and the year harvested. One study (*75*) reported a threefold variation in total isoflavone content among four varieties of soybeans, while a 30% variation was noted in a single variety of soybeans over a 4-year period. The content of individual isoflavones varied as much as 50%. Not surprisingly, location influences isoflavone content, even within fairly close geographical areas.

Duke noted that chemical variation is not limited to the isoflavones. In some instances as much as a fivefold variation was found among different phenolic acids in soybeans, many of which have also been investigated as potential anticarcinogens.

Isoflavones in Plant Physiology

Renee Kosslak described the role of isoflavones in defense strategies utilized by plants. Plants produce a wide range of products or secondary metabolites thought to enhance their survival (*76*). The isoflavones daidzein and genistein are the major inducers of the nodulation genes in *Bradyrhizobium* bacteria, which form nodules on soybeans (*77*).

The genetic regulation of isoflavone synthesis in plants is not well understood, in part because of the limited number of appropriate mutants affecting this pathway (*78,79*). In soybeans, near-isogenic lines that differ in their root fluorescence are being examined to determine whether they are active in genetic regulation of isoflavone synthesis (*80*). (These differences in root fluorescence in soybeans were first observed in 1934.) There are five loci that affect root fluorescence (*80*), and although specific substances responsible for this property have not been identified, isoflavones are thought to be involved. Preliminary data indicate that the levels of daidzein, genistein, and coumestrol, which is also a phytoestrogen, were either reduced or absent in root extracts from three of the nonfluorescent isolines tested (Kosslak R: unpublished observations).

Kosslak suggested that if future research implicates isoflavones and/or phytoestrogens as important dietary factors in cancer prevention and if the demand for soybean specialty products materializes, it may be possible to manipulate levels of these compounds in soybeans, using root fluorescence as a marker.

Soybean Processing

Bernie Szuhaj briefly discussed soybean processing procedures (*81-83*). Solvent extraction is the primary method of producing soybean products today. Soybeans entering the plant are first cleaned, cracked, and dehulled. Then moisture is added so they can be "flaked," leaving a product that is 3% hypocotyl, 89% cotyledon, and 8% hulls. The oil is removed from the flakes by hexane, producing defatted flakes and oil. From the defatted flakes come a variety of products. Soy protein content, on a dry weight basis, is 50% for soy flour and grits to about 70% for protein concentrates and about 90% for protein isolates. The difference between soy protein concentrates and isolates is larger percentage of carbohydrate in concentrates. Many commercial doughnuts in Europe and Asia, there is particular interest in fat soy flours for baking.

Szuhaj noted that most soybean products are used for animal feed, while the soy protein concentrates and isolates are marketed primarily for their multifunctional properties—emulsifying, gelling, fat binding, texturizing. Soy products play a major role in meat products, which are added to a wide variety of foods, from

meat products, such as ground beef, contain up to 25% soy. These products have been used in the Armed Forces' canteens since 1983 and in the federal school lunch program.

Discussion

This workshop had two objectives: *1*) to evaluate the relationship between the risk of certain cancers and consumption of soybeans, products derived from soybeans, and/or specific components of soybeans and 2) to recommend research initiatives aimed at clarifying this relationship. The consensus of the meeting was that there are sufficient data to justify studying the impact of soybean intake on cancer risk in humans.

There were three workshop recommendations. First, future dietary studies involving soybeans should be carried out using soy products rather than isolated compounds, since soybeans appear to contain several potential anticarcinogens. Additionally, because components of food interact, both negatively and positively, with each other, the potential benefit of soy products cannot be accurately predicted solely on the basis of the effects of individual soybean components. This does not, however, prohibit future use of isolated soybean components as chemopreventive agents in clinical trials. Second, standardized and improved analytical methods are needed so that the contents of all soy-based materials employed in soybean research, whether soybean fractions or soy products, can be accurately described. This methodology will allow for valid comparisons among studies. Third, basic research on the absorption, metabolism, and physiology of potential anticarcinogens in humans should be conducted. This research will likely help to determine the clinical relevancy of these compounds and to provide a basis for selecting specific soy products for use in future dietary studies.

References

(*1*) SETCHELL KDR: Naturally occurring non-steroidal estrogens of dietary origin. *In* Estrogens in the Environment (McLachlan JA, ed). New York: Elsevier, 1985, pp 69–85

(*2*) BRADBURY RB, WHITE DC: Oestrogens and related substances in plants. Vitam Horm 12:207–233, 1954

(*3*) FARNSWORTH NR, BINGEL AS, CORDELL GA, ET AL: Potential value of plants as sources of new antifertility agents. II. J Pharm Sci 64:717–754, 1975

(*4*) BENNETTS HW, UNDERWOOD EJ, SHEIR FL: A specific breeding problem of sheep on subterranean clover pastures in western Australia. Aust Vet J 22:2–12, 1946

(*5*) AXELSON M, DIRK DN, FARRANT RD, ET AL: The identification of the weak oestrogen equol[7-hydroxy-3-(4'–hydroxyphenyl)chroman] in human urine. Biochem J 201:353–357, 1982

(*6*) SETCHELL KDR, GOSSELIN SJ, WELSH MB, ET AL: Dietary estrogens—A probable cause of infertility and liver disease in captive cheetahs. Gastroenterology 93:225–233, 1987

(*7*) SETCHELL KDR, GOSSELIN SJ, WELSH MB, ET AL: Dietary factors in the development of liver disease and infertility in the captive cheetah. Presented at the International Symposium on Nutrition, Malnutrition, and Dietetics in Dogs and Cats, Hanover, Federal Republic of Germany, Sept 1987

(*8*) SETCHELL KDR, BOXRIELLO SP, HULME P, ET AL: Nonsteroidal estrogens of dietary origin: Possible roles in hormone-dependent disease. Am J Clin Nutr 40:569–578, 1984

(*9*) AXELSON M, SJOVALL J, GUSTAFSSON BE, ET AL: Soya — A dietary source of the non-steroidal oestrogen equol in man and animals. J Endocrinol 102:49–56, 1984

(*10*) AXELSON M, SETCHELL KDR: The excretion of lignans in rats — Evidence for an intestinal bacterial source for this new group of compounds. FEBS Lett 123:337–342, 1981

(*11*) SETCHELL KDR, WELSH MB, LIM CK: High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection. Amsterdam: Elsevier, 1987

(*12*) BARBUCH RJ, COUTANT JE, WELSH MB, ET AL: The use of thermospray liquid chromatography/tandem mass spectrometry for the class identification and structural verification of phytoestrogens in soy protein preparations. Biomed Environ Mass Spectrom 18:973–977, 1989

(*13*) SETCHELL KDR, ADLERCREUTZ H: Mammalian lignans and phytoestrogens — Recent studies on the formation, metabolism, and biological role in health and disease. *In* Role of the Gut Flora in Toxicity and Cancer. London: Academic Press, 1988, pp 315–345

(*14*) BARNES S, GRUBBS C, SETCHELL KDR, ET AL: Soybeans inhibit mammary tumors in models of breast cancer. *In* Mutagens and Carcinogens in the Diet (Pariza M, ed). New York: Wiley-Liss, 1990, pp 239–253

(*15*) NAGASAWA H: Nutrition and breast cancer: A survey of experimental and epidemiological evidence. IRCS J Med Sci 8:317–325, 1980

(*16*) AKIYAMA T, ISHIDA J, NAKAGAWA S, ET AL: Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262:5592–5595, 1987

(*17*) OKURA A, ARAKAWA H, OKA H, ET AL: Effects of genistein on topoisomerase activity and the growth of [val 12] Ha-*ras*-transformed NIH 3T3 cells. Biochem Biophys Res Commun 157:183–189, 1988

(*18*) LINASSIER C, PIER M, LE PECQ J-B, ET AL: Mechanisms of action in NIH 3T3 cells of genistein, an inhibitor of EGF receptor tyrosine kinase activity. Biochem Pharmacol 39:187–193, 1990

(*19*) SARIASLANI FS, KUNZ DA: Induction of cytochrome P-450 in *Streptomyces griseus* by soybean flour. Biochem Biophys Res Commun 141:405–410, 1986

(*20*) ELDRIDGE AC: Determination of isoflavones in soybean flours, protein concentrates, and isolates. J Agr Food Chem 30:353–355, 1982

(*21*) MURPHY PA: Phytoestrogen content of processed soybean products. Food Technol 36:62–64, 1982

(*22*) ERDMAN JW JR, FORDYCE EJ: Soy products and the human diet. Am J Clin Nutr 49:725–737, 1989

(*23*) ST CLAIR WH, BILLINGS PO, CAREW JA, ET AL: Suppression of dimethylhydrazine-induced carcinogenesis in mice by dietary addition of the Bowman-Birk protease inhibitor. Cancer Res 50:580–586, 1990

(*24*) MESSADI DV, BILLINOS P, SIIKLAR G, ET AL: Inhibition of oral carcinogenesis by a protease inhibitor. JNCI 76:447–452, 1986

(*25*) WITSCHI H, KENNEDY AR: Modulation of lung tumor development in mice with the soybean-derived Bowman-Birk protease inhibitor. Carcinogenesis 10:2275–2277, 1989

(*26*) YAVELOW J, COLLINS M, BIRK Y, ET AL: Nanomolar concentrations of Bowman-Birk soybean protease inhibitor suppress x-ray-induced transformation in vitro. Proc Natl Acad Sci USA 82:5395–5399, 1985

(*27*) KENNEDY AR, LITTLE JB: Effects of protease inhibitors on radiation transformation in vitro. Cancer Res 41:2103–2108, 1981

(*28*) KENNEDY AR: The conditions for the modification of radiation transformation in vitro by a tumor promoter and protease inhibitors. Carcinogenesis 6:1441–1445, 1985

(*29*) KENNEDY AR, BILLINGS PC: Anticarcinogenic actions of protease inhibitors. *In* Anticarcinogenesis and Radiation Protection (Corutti PA, Nygaard OF, Simic MG, eds). New York: Plenum, 1987, pp 285–295

(*30*) BIRK Y: Protease inhibitors of plant origin and role of protease inhibitors in human nutrition. *In* Protease Inhibitors as Potential Cancer Chemopreventive Agents (Troll W, Kennedy AR, eds). New York: Plenum, In press

(*31*) MILLS PK, BEESON WL, ABBEY DE, ET AL: Dietary habits and past medical history as related to fatal pancreas cancer risk among Adventists. Cancer 61:2578–2585, 1988

(*32*) GUMBMANN MR, SPANGLER WL, DUGAN GM, ET AL: Safety of trypsin inhibitors in the diet: Effects on the rat pancreas of long-term feeding of soy flour and soy protein isolate. *In* Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods (Friedman M, ed). New York: Plenum, 1986, pp 33–79

(*33*) BRANDON DL, BATES AH, FRIEDMAN M: Enzyme-linked immunoassay of soybean Kunitz trypsin inhibitor using monoclonal antibodies. J Food Sci 53:97–101, 1988

(*34*) BRANDON DL, BATES AH, FRIEDMAN M: Monoclonal antibody-based enzyme immunoassay of Bowman-Birk protease inhibitor of soybeans. J Agr Food Chem 37:1192–1196, 1989

(*35*) HYMOWITZ T: Genetics and breeding of soybeans lacking the Kunitz trypsin inhibitor. *In* Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods (Friedman M, ed). New York: Plenum, 1986, pp 291–298

(*36*) FRIEDMAN M, BRANDON DL, BATES AH, ET AL: Comparison of a commercial soybean cultivar and an isoline lacking the Kunitz trypsin inhibitor: Composition, nutritional value, and effects of heating. J Agr Food Chem. In press

(*37*) DIPIETRO CM, LIENER IE: Heat inactivation of the Kunitz and Bowman-Birk soybean protease inhibitors. J Agr Food Chem. In press

(38) Birk Y: The Bowman-Birk Inhibitor. Trypsin- and chymotrypsin-inhibitor from soybeans. Int J Pept Protein Res 25:113–131, 1985
(39) Oste RE, Brandon DL, Bates AH, et al: Effect of Maillard browning reactions of the Kunitz soybean trypsin inhibitor on its interaction with monoclonal antibodies. J Agr Food Chem 38:258–261, 1990
(40) Brandon DL, Bates AH, Friedman M: Antigenicity of soybean protease inhibitors. *In* Protease Inhibitors as Potential Cancer Chemopreventive Agents (Troll W, Kennedy AR, eds). New York: Plenum. In press
(41) Liener IE, Kakade ML: Protease inhibitors. *In* Toxic Constituents of Plant Foodstuffs (Liener IE, ed), 2nd ed. New York: Academic Press, 1980, pp 7–71
(42) McGuiness EE, Morgan RG, Levison DA, et al: The effects of long-term feeding of soya flour on the rat pancreas. Scand J Gastroenterol 15:497–502, 1980
(43) Morgan RGH, Levison DA, Hopwood D, et al: Potentiation of the action of azaserine on the rat pancreas by raw soya bean flour. Cancer Lett 3:87–90, 1977
(44) Liener IE, Nitsan Z, Srisangnam C, et al: The USDA Trypsin Inhibitor Study. II. Time-related biochemical changes in the pancreas of rats. Qual Plant Foods Hum Nutr 35:243–258, 1985
(45) Schneeman BO, Gallaher D: Pancreatic response to dietary trypsin inhibitor: Variations among species. Adv Exp Med Biol 199:185–187, 1986
(46) Nitsan Z, Liener IE: Enzymic activities in the pancreas, digestive tract, and feces of rats fed raw or heated soy flour. J Nutr 106:300–305, 1976
(47) Liddle RA, Goldfine ID, Williams JA: Bioassay of plasma cholecystokinin in rats: Effects of food, trypsin inhibitor, and alcohol. Gastroenterology 87:542–549, 1984
(48) Liener IE, Goodale RL, Deshmukh A, et al: Effect of a trypsin inhibitor from soybeans (Bowman-Birk) on the secretory activity of the human pancreas. Gastroenterology 94:419–427, 1988
(49) Calam J, Bojarski JC, Springer CJ: Raw soya bean flour increases cholecystokinin release in man. Br J Nutr 58:175–179, 1987
(50) Harwood JL, Russell NJ: Lipids in Plants and Microbes. London: George Allen and Unwin, 1984, p 23
(51) Weihrauch JL, Gardner JM: Sterol content of foods of plant origin. J Am Diet Assoc 73:39–47, 1978
(52) Nair PP, Turjman N, Kessle G, et al: Diet, nutrition intake, and metabolism in populations at high and low risk for colon cancer: Dietary cholesterol β-sitosterol, and stigmasterol. Am J Clin Nutr 40:927–930, 1984
(53) Hirai K, Shimazu C, Takezoe R, et al: Cholesterol, phytosterol and polyunsaturated fatty acid levels in 1982 and 1957 Japanese diets. J Nutr Sci Vitaminol (Tokyo) 32:363–372, 1986
(54) Oakenfull DG: Saponins in food — A review. Food Chem 6:19–40, 1981
(55) Raicht RF, Cohen BI, Fazzini EP, et al: Protective effect of plant sterols against chemically induced colon tumors in rats. Cancer Res 40:403–405, 1980
(56) Deschner EE, Cohen BI, Raicht RF: The kinetics of the protective effect of β-sitosterol against MNU-induced colonic neoplasia. J Cancer Res Clin Oncol 103:49–54, 1982
(57) Oakenfull DG, Fenwick DE, Hood RL, et al: Effect of saponins on bile acids and plasma lipids in the rat. Br J Nutr 42:209–216, 1979
(58) Bomford R: Studies on the cellular site of action of the adjuvant activity of saponin for sheep erythrocytes. Int Arch Allergy Appl Immunol 67:127–131, 1982
(59) Maharaj I, Froh KJ, Campbell JB: Immune responses of mice to inactivated rabies vaccine administered orally: Potentiation by Quillaja saponin. Can J Microbiol 32:414–420, 1986
(60) Huang H-P, Cheng C-F, Lin WQ, et al: Antitumor activity of total saponins from *Dolichos falactus* Klein. Acta Pharmacol Sinica 3:386, 1982
(61) Yindi Z: Effects of astragulus saponin-I on cAMP and cGMP levels in plasma and DNA synthesis in regenerating liver. Yao Hsueh Hsueh Pao 19:619, 1984
(62) Aswal BS, Bhakuni AK, Kar K, et al: Screening of Indian plants for biological activity. Part X. Indian J Exp Biol 22:312–332, 1984
(63) Sati OP, Pant G, Nohara T, et al: Cytotoxic saponin from asparagus and agave. Pharmazie 40:586, 1985
(64) Tokuda H: Inhibitory effects of 12-*O*-tetradecanoylphorbol-13-acetate and teleocidin-B-induced Epstein-Barr virus by saponin and its related compounds. Cancer Lett 40:309–317, 1988
(65) Graf E, Eaton JW: Dietary suppression of colonic cancer. Fiber or phytate? Cancer 56:717–718, 1985
(66) Graf E, Eaton JW: Antioxidant functions of phytic acid. Free Radic Biol Med 8:61–69, 1990
(67) Graf E, Mahoney JR, Bryant RG, et al: Iron-catalyzed hydroxyl formation. J Biol Chem 259:3620–3624, 1984
(68) Jariwalla RJ, Sabin R, Lawson S, et al: Effects of dietary phytic acid (phytate) on the incidence and growth rate of tumors promoted in Fischer rats by a magnesium supplement. Nutr Rev 8:813–827, 1988
(69) Shamsuddin AM, Elsayed AM, Ullah A: Suppression of large intestinal cancer in F344 rats by inositol hexaphosphate. Carcinogenesis 9:577–580, 1988
(70) Baten A, Ullah A, Tomazic VJ, et al: Inositol-phosphate-induced enhancement of natural killer cell activity correlates with tumor suppression. Carcinogenesis 10:1595–1598, 1989
(71) Shamsuddin AM, Ullah A: Inositol hexaphosphate inhibits large intestinal cancer in F344 rats 5 months after induction by azoxymethane. Carcinogenesis 10:625–626, 1989
(72) Shamsuddin AM, Ullah A, Chakravarthy AK: Inositol and inositol hexaphosphate suppress cell proliferation and tumor formation in CD-1 mice. Carcinogenesis 10:1461–1463, 1989
(73) Baten A, Shamsuddin A: Inhibition of cell growth and induction of differentiation in K-562 human erythroleukemia cell lines by inositol hexaphosphate. Proc Am Assoc Cancer Res 30:182, 1989
(74) Duke JA: Handbook of Nuts. Boca Raton, FL: CRC Press, 1989, pp 1–343
(75) Eldridge AC, Kwolek WF: Soybean isoflavones: Effect of environment and variety of composition. J Agr Food Chem 31:394–396, 1983
(76) Williams DH, Stone MJ, Hauck PR, et al: Why are secondary metabolites (natural products) biosynthesized? J Nat Prod 52:1189–1208, 1989
(77) Kosslak RM, Bookland R, Barkei J, et al: Induction of *Bradyrhizobium japonicum* common in nod genes by isoflavones isolated from *Glycine max*. Proc Natl Acad Sci USA 84:7428–7432, 1987
(78) Dewick PM: Isoflavonoids. *In* The Flavonoids: Advances in Research Since 1980 (Harborne JB, ed). London: Chapman and Hall, 1988, pp 125–210
(79) Dixon RA, Bailey JA, Bell JN, et al: Rapid changes in gene expression in response to microbial elicitation. Philos Trans R Soc Lond [Biol] B314:411–426, 1986
(80) Sawada S, Palmer RG: Genetic analyses of non-fluorescent root mutants induced by mutagenesis in soybeans. Crop Sci 27:62–65, 1987
(81) Smith AK, Circle SJ: Soybeans: Chemistry and Technology, vol 1, Proteins. Westport, Conn: AVI, 1972
(82) Soy Protein Council: Soy Protein Products — Characteristics, Nutritional Aspects and Utilization. Washington, DC: Soy Protein Council, 1987
(83) Campbell MF, Kraut CW, Yackel WC, et al: Soy protein concentrate. *In* New Protein Foods: Seed Storage Proteins (Altschul AM, Wilcke HL, eds), vol 5, chap 9. Orlando, Fla: Academic Press, 1981

D10

Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet[1-4]

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ABSTRACT Epidemiologic studies revealed low mortality in hormone-dependent cancer in Japanese women and men consuming a traditional diet. We previously found that certain diphenolic food components, lignans and isoflavonoids, which are converted to biologically active hormone-like substances by intestinal microflora, may be cancer-protective agents. Therefore, we studied urinary excretion of these compounds (enterolactone, enterodiol, daidzein, equol, and *O*-desmethylangolensin) in 10 women and 9 men in a rural village south of Kyoto, Japan. The subjects consumed a typical low-fat diet with much rice and soy products, fish, and vegetables. An isotope-dilution gas chromatographic–mass spectrometric method was used for the assays. The urinary excretion of lignans was low but that of the isoflavonoids was very high. The excretion of isoflavonoids correlated with soybean-product intake. The low mortality in breast and prostate cancer of Japanese women and men, respectively, may be due to the high intake of soybean products. *Am J Clin Nutr* 1991;54:1093–1100.

KEY WORDS Japanese, diet, urine, lignans, isoflavonoids, enterolactone, enterodiol, daidzein, equol, genistein, *O*-desmethylangolensin, soybean, gas chromatography, mass spectrometry, sex-hormone–binding globulin

Introduction

Mammalian lignans and isoflavonoid phytoestrogens, occurring in all studied animal and human biological fluids and in feces, are diphenolic compounds with molecular weights similar to those of steroid estrogens (1–3). Precursors in plants seem to occur as glycosides (4, 5), and the mammalian compounds are produced from plant lignans and isoflavonoids by intestinal microflora (6–8). Most of the original plant aglycones, such as formononetin, matairesinol, and secoisolariciresinol, occur only in very low concentrations in urine (9, 10). All compounds investigated so far are weakly estrogenic but have shown many other biological activities, producing antiestrogenic (1–3); antiviral (11, 12); and antiproliferative, cytotoxic, and growth-inhibiting effects (3, 13–15). Studies indicate that they most likely stimulate the production of sex-hormone–binding globulin (SHBG) in the liver (2, 14–18) and may in this way significantly influence biological activity of the sex hormones. The higher SHBG values seen in vegetarians (2, 17–19) are probably due to the effect of these diphenolic compounds on liver synthesis of the protein (14). Studies in both young and old women with breast cancer and in various dietary groups indicate that urinary excretion of these compounds is highest in vegetarians and lower in omnivores and breast-cancer patients (2, 18, 20). It was shown that their urinary excretion correlates with the intake of fiber-rich food (2, 17, 18).

Japanese women and women of Japanese origin in Hawaii consuming a diet similar to the original traditional Japanese diet have low breast-cancer incidence and mortality (21–24). Similarly, Japanese men have low mortality with prostate cancer, although autopsy studies have found that the incidence of prostate cancer in Japanese and Western men are similar (25–27). These cancers are sex-hormone dependent and could potentially be influenced both by alterations of sex-hormone metabolism caused by lignans and isoflavonoids or by a direct effect of these compounds on their growth. Because of the associations between diet and these diseases, we decided to study the urinary excretion of lignans and isoflavonoid phytoestrogens in groups of Japanese men and women consuming a traditional diet. A preliminary report was published as an abstract (28).

Subjects and methods

Participants

The subjects participating in this investigation were apparently healthy and were recruited in a small rural village south of Kyoto,

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[2] Preliminary report published as an abstract.

[3] Supported by Sigrid Jusélius and Finnish Cancer Foundations and the Medical Research Council of the Academy of Finland.

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Received January 4, 1991.
Accepted for publication April 17, 1991.

Japan. Two of the women were found to have hypertension (blood pressure 146/96 and 180/100, respectively). Most of the participants were farmers cultivating tea and rice. Originally 10 men and 10 women volunteered for the study, but 1 man was dropped because his urine volume was not known. Their main work was in agriculture and they consumed mainly their own products. The ages of the men and women were 50.4 ± 18.0 and 46.8 ± 11.5 y, respectively. Height, weight, and body mass index [BMI, in weight (kg)/height $(m)^2$] were, respectively, 160.8 ± 7.8 cm, 58.6 ± 5.8 kg, and 22.7 ± 2.3 for men and 153.1 ± 6.5 cm, 52.9 ± 7.2 kg, and 22.6 ± 3.5 for women. All subjects were within 15% of normal weight.

Collection of samples

Urine was collected for 48 h in plastic bottles containing 2 g ascorbic acid. The bottle was kept in a cool place during collection. The urine was mixed and measured and a sample was frozen as soon as possible and transported to Finland in dry ice for analysis.

Dietary data

The study was carried out in October 1985. Before the survey a nutritionist explained how to weigh the food components and how to write down the results on a form. Most of the food was weighed. Some food, such as bread and milk, was recorded as a piece of bread or cup of milk and the nutritionist estimated the weight of these food items afterwards. Food intake was recorded for 3 d and the nutritionist followed all subjects every day during the survey period. Calculation of the food data was made by an experienced nutritionist using the *Standard Tables of Food Composition in Japan* (29); for fiber calculations the *Food Composition Tables of Dietary Fibers, Minerals, Cholesterol, Fatty Acids* was used (30). The amount of soy sauce in the diet was calculated from the total sodium chloride content of the urine. According to earlier studies Japanese obtain 25.8% of their sodium chloride from soy sauce (31). Soy sauce contains 15% NaCl. The consumption of soy sauce is estimated by using the following formula:

$$\text{Soy sauce} = (\text{amount of NaCl in urine}) \times 0.258/0.15$$

This is the traditional way to estimate soy sauce consumption in Japanese subjects because they do not add any other salt to their food. It is an estimate and not an exact figure and the values were not included in the correlation analyses.

Analytical method

The trivial and systematic names of the compounds measured and discussed are as follows [structures were shown previously (3)]: enterolactone (Enl), *trans*-2,3-bis[(3-hydroxyphenyl)methyl]-γ-butyrolactone; enterodiol (End), 2,3-*bis*[(3-hydroxyphenyl)-methyl]-butane-1,4-diol; daidzein (Da), 4',7-dihydroxyisoflavone; equol (Eq), 4',7-dihydroxyisoflavan; *O*-desmethylangolensin (*O*-Dma), 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-propan-1-one.

The method used was a modification of a method for determining the estrogen profile in urine by ion-exchange chromatography and capillary gas chromatography–mass spectrometry in the selected ion-monitoring mode (GC-MS-SIM, or GC/MS) (32–34). Originally, estrogens also were determined but because of very low concentrations of some fractions, the amount of urine saved for the purpose was too small and the analyses could not be repeated. Therefore, only the lignan and isoflavonoid values are presented. Only modifications of the method are described.

Protection of the carbonyl functions by ethoximation (necessary only for the estrogens), and extraction with a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) were carried out as described (33, 34). The removal of inhibitors of the enzyme hydrolysis by ion-exchange chromatography on a DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) column in the acetate form was done in a smaller column (0.5 × 3 cm instead of 0.5 × 5 cm). For hydrolysis and purification of the hydrolysate, before evaporation of the last fraction obtained from the above DEAE-Sephadex column, the following deuterated internal standards were added to the eluate: d_6-Enl and -End, d_4-Da and -Eq, and d_5-*O*-Dma (35, 36). This was followed by hydrolysis and Sep-Pak extraction; application of the methanolic extract directly on the QAE-Sephadex A-25 in the acetate form (0.5 × 5 cm); and elution of the estrogens, lignans, and Eq with 4 mL methanol as described. The modification in this step is that *O*-Dma and Da are eluted after this with 4 mL 0.2 mol acetic acid/L in methanol. This fraction is then, after evaporation of the solvent, ready for derivatization (trimethylsilyl ethers) and GC/MS. Selective fractionation of estrogens with vicinal *cis*-hydroxyls was carried out in a borate column with new dimension (0.5 × 3 cm instead of 0.5 × 2.5 cm). Elution of the diphenols was carried out as described and this fraction contains the isoflavan Eq and the two lignans Ent and End.

The two fractions containing lignans and isoflavonoid phytoestrogens and their deuterated internal standards are converted to their trimethylsilyl ether (TMS) derivatives (32) and quantified by GC/MS by using the following ion pairs (mass/charge): Eq, 386/390; Da, 398/402 (and 383/387); End, 410/416; Enl, 442/448; and *O*-Dma, 459/464 (36). The measurements were carried out with a Hewlett-Packard 5995 B GC/MS (Avondale, PA) instrument equipped with a Pascal work station and with an automatic injector.

Urinary excretion of < 0.0025 μmol/d cannot be measured, and between 0.0025 and 0.005 μmol/d the method must be regarded as semiquantitative. The mean values and interassay imprecision for the control pooled-urine sample, measured 59 times in single assays during 1 y, were as follows: Enl, 3.65 μmol/d (CV 7.4%); End, 0.364 μmol/d (CV 11.6%); and Eq, 0.042 μmol/d (CV 9.4%). For Da at a concentration of 0.028 μmol/d, the interassay imprecision is 11.0% (n = 14) and for *O*-Dma at the high concentrations in this study, the interassay imprecision is 8–10% (CV).

The samples were analyzed in two batches and the values for the control sample were almost identical both times and the same as in analyses before and after these two batches.

Statistical methods

The food data are presented as arithmetic means (±SD) and the lignan and phytoestrogen results as arithmetic means (±SD) and geometric means. Geometric means were used when necessary because of skewness of the distribution of the results. The statistical analyses were carried out by using the *StatView* program for Macintosh (Abacus Concepts, Berkeley, CA). The degree of univariate associations between two variables were estimated as Pearson's correlation coefficients (r). The pairs of

TABLE 1
Intake of various food stuffs by the Japanese women and men consuming a traditional Japanese diet*

Nutrient	Women (n = 10)	Men (n = 9)
	g/d	
Rice	578.5 ± 222.5	764.7 ± 240.3
Wheat	59.5 ± 46.0	139.0 ± 113.6
Potato	62.6 ± 30.2	55.2 ± 34.6
Sugar	8.1 ± 7.0	8.1 ± 7.4
Fats	13.1 ± 7.6	12.7 ± 6.9
Pulses and beans	56.5 ± 36.0	40.9 ± 32.0
Fruit	228.2 ± 111.9	146.9 ± 114.0
Green and yellow vegetables	60.6 ± 33.3	55.7 ± 35.2
Other vegetables	139.3 ± 69.3	130.9 ± 77.2
Pickles	32.9 ± 24.9	23.2 ± 21.2
Algae	1.8 ± 2.0	0.7 ± 0.7
Fish	98.7 ± 46.6	113.6 ± 56.5
Meat	37.0 ± 30.1	73.6 ± 58.4
Eggs	38.4 ± 16.6	57.4 ± 30.6
Milk	112.7 ± 131.0	90.9 ± 90.2
Beer	5.1 ± 16.1	454.6 ± 647.1

* $\bar{x}$ ± SD.

adjusted group means for the two groups studied (women and men) were compared by nonpaired t test.

Results

The intake of various types of food are shown in Table 1, and Table 2 shows the results of the calculations with regard to energy;

TABLE 2
Energy intake, intake of various nutrients, and some ratios in the two study groups*

Nutrient	Women (n = 10)	Men (n = 9)
Energy		
(MJ/d)	8.29 ± 1.64	10.79 ± 3.48
(kcal/d)	1973 ± 391	2569 ± 829
Animal protein (g/d)	35.3 ± 13.9	47.8 ± 18.9
Vegetable protein (g/d)	38.2 ± 10.1	45.1 ± 10.6
Total protein (g/d)	73.6 ± 12.2	93.0 ± 28.4
Carbohydrates (g/d)	311.4 ± 77.0	383.3 ± 100.6
Total fat (g/d)	44.4 ± 14.4	51.0 ± 25.9
Total fiber (g/d)	16.9 ± 4.9	15.3 ± 6.0
Animal protein (%)†	47.2 ± 15.9	49.8 ± 7.9
Proteins (%)‡	15.2 ± 2.1	14.6 ± 1.5
Carbohydrates (%)‡	64.6 ± 6.8	68.2 ± 5.1
Fats (%)‡	20.3 ± 5.5	17.2 ± 4.9
Fat (g/kg body wt)	0.86 ± 0.31	0.85 ± 0.37
Fiber		
(mg/J)	2.1 ± 0.7	1.5 ± 0.7
(g/1000 kcal)	8.8 ± 3.0	6.4 ± 3.0
Fiber (g/kg body wt)	0.33 ± 0.10	0.26 ± 0.09
Fat-fiber ratio	2.5 ± 0.9	2.4 ± 0.9

* $\bar{x}$ ± SD.
† Percent of total protein.
‡ Percent of energy.

TABLE 3
Dietary intake of soy products by the two groups studied*

Soy product	Women (n = 10)	Men (n = 9)
	g/d	
Tofu (soybean curd)	25.0 ± 22.9	18.7 ± 28.8
Miso (bean paste)	12.5 ± 6.2	8.5 ± 6.4
Aburaage (fried thin tofu)	2.6 ± 3.6	3.7 ± 4.2
Atuage (fried thick tofu)	4.0 ± 12.7	0.8 ± 2.3
Koridofu (dried soybean curd)	0.37 ± 0.78	0.07 ± 0.2
Fermented soybeans	2.4 ± 4.5	0.9 ± 2.8
Boiled beans	7.7 ± 17.8	6.5 ± 7.8
Soy sauce	22.9 ± 6.1	19.2 ± 4.7
Soy products (sauce excluded)	54.4 ± 34.3	39.2 ± 36.4

* $\bar{x}$ ± SD.

animal and vegetable protein; total proteins, carbohydrates, fats, and fiber; percentage animal protein and percentage protein; and carbohydrate and fat as percent of total calories. Furthermore, we calculated the fat intake per kilogram body weight, fiber intake per J (per 1000 kcal), and the fat-fiber ratio (Table 2). The diet was a low-fat (fat 17.2% and 20.3% of total calories for men and women, respectively), low-animal-protein diet with moderate amounts of fiber and a low fat-fiber ratio, which is typical for the traditional Japanese diet (37).

Table 3 shows the dietary intake of soy products, which were expected to be the most important source of precursors for the urinary isoflavonoids (3).

Table 4 shows the mean excretion values for the two lignans and three isoflavonoid phytoestrogens. The results show a relatively low excretion of enterolactone, a normal excretion for enterodiol, and a very high excretion of isoflavonoid phytoestrogens. The individual results showed large variation, particularly for equol (from 0 to 10.95 μmol/d). For comparison note that the geometric mean values in young omnivorous women living in Helsinki and in Boston for enterolactone, enterodiol, daidzein, equol, and *O*-desmethyl-angolensin were 2.46, 0.20, 0.22, 0.10, 0.03, and 2.05, 0.28, 0.32, 0.07, and 0.03 μmol/d, respectively (2).

TABLE 4
Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese women and men consuming traditional Japanese diet*

Compound	Women (n = 10)	Men (n = 9)
	μmol/d	
Enterolactone	1.4 ± 1.4 (0.89)	1.1 ± 0.7 (0.89)
Enterodiol	0.7 ± 1.3 (0.41)	0.4 ± 0.3 (0.22)
Total lignans	2.1 ± 2.6 (1.38)	1.5 ± 0.9 (1.13)
Daidzein	2.6 ± 4.0 (2.55)	2.2 ± 2.0 (1.45)
Equol	2.6 ± 4.0 (0.56)	3.0 ± 4.6 (0.54)
O-desmethylangolensin	0.7 ± 0.6 (0.51)	0.2 ± 0.3 (0.11)
Total isoflavonoids	6.9 ± 6.8 (4.73)	3.9 ± 3.3 (2.57)
Total diphenols	9.1 ± 9.3 (6.7)	5.4 ± 4.0 (4.1)

* $\bar{x}$ ± SD (geometric $\bar{x}$).

Table 5 presents a correlation matrix of various food components and urinary excretion of lignans and isoflavonoids in the total material of 19 subjects for whom both food and phytoestrogen data were available.

Discussion

In a previous study of oriental immigrant women from southeast Asia residing in Hawaii (38), the diet was similar to that consumed by the men and women in the rural village in Japan. In the present study the women had a greater energy intake (an additional ~2.1 MJ/d, or 500 kcal/d), which may be due to a physically more active life. However, the percentage intake of calories as fat and the dietary fiber and fat-fiber ratio were very similar to the corresponding values in the previous study. Except for the energy intake the values are very different from those seen in Western societies where the fiber intake is similar but the fat-fiber ratio is much higher. Women living in the Boston area had a fat-fiber ratio of 7.7 for the premenopausal women and 4.6 for the postmenopausal women compared with 2.5 for the women in the present study (39).

With regard to protein intake, expressed as g/d and as percentage of calories, the mean values in the present study were similar and slightly lower, respectively, than those of the immigrants from southwest Asia (38).

Our results are in good agreement with those from an earlier study of 300 female agricultural workers from 18 regions in Japan (37) except for dietary fiber intake, which was much lower (between 5 and 6 g/d) in the women in the earlier study (which may represent crude fiber intake). However, according to the national nutrition survey in Japan, the dietary fiber intake was 22.8 g/d in 1951 and decreased year by year to 17.4 g/d in 1985. These figures are in better agreement with our results obtained in 1985, which show a mean dietary fiber intake in the whole group of ~16 g/d. This latter value is also in good agreement with the value of 13 g/d for nonstarch polysaccharides found by analyses of the Japanese diet in another study (40). On the basis of these investigations and the present investigation, it may be concluded that the amount of dietary fiber in a traditional oriental diet is comparable with that in many Western societies (38–40). We may also conclude that the diet of our subjects was typical for a rural area, where the people to a large extent consume their own products and have a traditional Japanese diet.

The urinary excretion of Enl was, with few exceptions, low in both men and women (Tables 4 and 1A) and was the same as found for the postmenopausal breast-cancer patients in Boston (20). We found a weak correlation between intake of green and yellow vegetables and excretion of Enl and total lignans (Table 5) but no correlation with rice intake. Because these subjects consumed large amounts of rice, it seems justified to conclude that refined rice contains very low amounts, if any, of lignan precursors. There was a better correlation with the intake of soybeans, which thus also may be a source of Enl precursors (Table 5). It is known that soy sauce contains coniferyl alcohol the building block for lignans and lignin (41). The excretion of the lignan End was also found to be associated with the intake of beans and pulses and soy products in general (Table 5).

The excretion of the isoflavonoid phytoestrogens is very high in these Japanese men and women compared with values obtained in women living in Boston (2, 20) and in the Helsinki area (2, 18). The Japanese women in the present study excrete 10 times more Da and 20–30 times more Eq and *O*-Dma than did omnivorous and lactovegetarian women living in the abovementioned two cities. Of the 19 subjects, 47% and 89% excrete micromole amounts of Eq and Da per day, respectively, a phenomenon very rarely seen in subjects consuming a Western diet but seen in subjects consuming a macrobiotic diet (2). The values in an additional study group of nine subjects, including three children (*see* Appendix A), were not significantly different from those in the two main groups (Tables 4 and 1A); they were in fact surprisingly identical. The excretion of matairesinol, the precursor lignan for enterodiol, was very low, but genistein excretion was very high. Genistein is the center of interest in many laboratories because of its very interesting antiproliferative and

TABLE 5
Correlation matrix of various food components and urinary excretion of ligans and isoflavonoids in the whole material (n = 19)

Nutrient	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Total isoflavonoids	Total diphenols
Green and yellow vegetables	0.525*		0.460*					
Pulses and beans		0.541*	0.492*	0.679†	0.737†	0.617†	0.668†	0.693†
Algae				0.561*			0.450‡	0.430‡
Total fat					0.584†			
Percent fat calories					0.469*			
Fat-fiber ratio					0.507*			
Meat					0.507*			
Soy products (not sauce)		0.481*		0.583†	0.746§	0.601†	0.585†	0.588†
Boiled soybeans	0.758§	0.892§	0.849§	0.632†	0.693§		0.757§	0.801§

* $P < 0.05$.
† $P < 0.01$.
‡ $0.05 < P < 0.10$.
§ $P < 0.001$.

antimitogenic effects (*see* below); genistein showed the highest concentration of all phytoestrogens in urine in these nine subjects. The mean value was almost 6 μmol/d and a value as high as 15.5 μmol/d was observed. Also in this smaller group most variation in the excretion values was found for Eq (from 0.01 to 9.16 μmol/d). In 21.4% of all subjects, equol excretion did not significantly differ from zero: this group included two of the three children; the mother of these two children did not excrete equol in significant amounts.

The low excretion of Enl in the Japanese subjects compared, eg, with Finnish women (2), is most likely due to low intake of grain (whole-grain) products such as bread (2, 17, 18, 42, 43). The precursors of the mammalian lignans seem to be located in the aleuronic layer of the grain close to the fiber (15) but definite evidence for this location has not yet been obtained. The mean End values are similar to those observed in lactovegetarian American and Finnish women and higher than in the omnivorous women from the same countries (2, 20). It is likely that the majority of the lignans in these Japanese subjects is derived from nongrain plant products (pulses and beans), as suggested by the correlations found in Table 5.

Eq excretion correlated positively with the intake of total fat ($P < 0.01$), fat-fiber ratio ($P < 0.05$), and meat ($P < 0.05$) and deviated in this aspect from all the other isoflavonoids. Some subjects are not able to produce Eq at all, as also shown previously for nonJapanese subjects (44). It is possible that those consuming more fat and meat have an intestinal flora more capable of producing Eq from Da, known to occur in large amounts in soybeans (45). Algae may also be a source of isoflavonoids because a positive correlation was found with Da ($r = 0.56$; $P < 0.05$) and total isoflavonoids ($r = 0.45$; $0.05 < P < 0.10$, NS). Algae were suggested to contain factors protective against breast cancer (46).

Lignans and bioflavonoids are candidates for a role as cancer-protective agents (2, 14–16) and as steroid competitors for various enzymes (47). Enl inhibits the aromatase enzyme and competes with the natural substrate androstenedione for the binding site on the cytochrome P450 enzyme (H Adlercreutz, C Bannwart, LE Vickery, et al, unpublished observations, 1985). Phytoestrogens and lignans (48; H Adlercreutz, Y Mousavi, J Clark, et al, unpublished observation, 1987) show interaction with estrogen receptors and flavonoids have antiproliferative effects on the human-breast-carcinoma cell line ZR-75-1 (49). Genistein is a very specific inhibitor of the tyrosine-specific protein kinases (50–55) and platelet-activating-factor–stimulated platelet aggregation, phospholipase C, and tyrosine kinase activity (56). Tyrosine kinase is an important mediator of the effects of some biologically important growth factors such as epidermal growth factor, insulin, platelet-derived growth factor, and insulin-like growth factor on cells. The flavonoids and lignans bind to the type II estrogen-binding sites (15, 57), now also called the bioflavonoid receptor (47, 58), and may in this way regulate by inhibition cell growth and proliferation of hormone-dependent cancers (58). Enzymes metabolizing bioflavonoids and steroids show structurally close similarity (47), indicating that they have the same origin. Furthermore, the isoflavonoid coumestrol complements, as does estradiol, the topography of spaces between base pairs in unwound DNA and simultaneously hydrogen-bond phosphate moieties on opposite strands (59).

One of the most important biological effects of the lignans and isoflavonoids seems to be their stimulation of SHBG synthesis in the liver (2, 14, 16–18). A high SHBG concentration leads to decreased metabolic clearance rate for the sex hormones and lower biological activity. However, Japanese and British women were found to have the same SHBG total-binding capacity, even though Japanese women bound relatively more estradiol to SHBG. This was suggested to be a result of lower affinity of albumin for estradiol in these women (60). It is possible that the phytoestrogens in the high amounts occurring in Japanese women could compete with estradiol for the albumin-binding sites and in this way lead to relatively more binding to SHBG.

SHBG concentrations tend to be lower in breast-cancer patients, particularly in postmenopausal women, and this seems at least partly to be due to diet (15). SHBG-binding capacity was significantly smaller in postmenopausal but not in premenopausal Japanese subjects with breast cancer compared with Japanese control subjects (61), agreeing with our own more recent results in American postmenopausal (43) women. Finnish premenopausal women with breast cancer did not differ in this respect from omnivorous control subjects but they had lower SHBG than did lactovegetarian women (18). Diet seems to be a much more important risk factor for postmenopausal than for premenopausal breast cancer (15). Miso (Japanese soybean paste) (62) or powdered soybean chips (63) (both before and after denaturation of the protease inhibitors) showed a tendency to decrease mammary-tumor formation and growth rate in rat breast-cancer models and soybean diet also reduced breast-tumor incidence in irradiated rats (64). This agrees with the slower average growth rate of postmenopausal breast cancers in Japanese compared with caucasian women in Hawaii (65).

The high concentration of phytoestrogens in the urine of Japanese men could be protective with regard to prostate cancer. Both lignans and isoflavonoids have estrogenic effects in numerous biological systems and may, because of this property, inhibit development of prostatic cancer. It is well known that in Japan and some other Asian countries, despite the same incidence of latent small or noninfiltrative prostatic carcinomas as in Western societies, the mortality is low (25–27). The high exogenous phytoestrogen concentrations could inhibit the growth of the latent carcinomas, postponing their development and making it more likely that the subjects die from some other disease (theory proposed in 1985) (66). Furthermore, the inhibitory effect of genistein on tyrosine-specific protein kinases of certain growth-factor receptors could play an important role. Decreased risk of prostate cancer is seen in Seventh-day Adventist men (67) consuming much beans, lentils, and peas and some dried fruits (rich sources of bioflavonoids) and in men of Japanese ancestry in Hawaii consuming much rice (mainly starch, which has some fiber-like effects in the gut) and tofu (68), supporting the view that these compounds are protective. Recently, Santti's group in Turku, Finland, in a collaborative study with us, observed that dietary soy prevented the development of precancerous changes in a neonatally estrogenized mouse used as a model for prostatic cancer (69), showing that dietary factors may already be important in the fetal and neonatal periods. This study and our observation of high phytoestrogen excretion in urine of children is important because they suggest that these compounds may change the endocrine milieu at the cellular level both in the neonatal period and in prepubertal and adolescent children. Thus, the results cited above and discussed more

extensively elsewhere (14, 15) speak for a role of the diphenols as cancer-protective substances.

It is concluded that Japanese subjects excrete very large amounts of isoflavonoids in urine, mainly genistein, daidzein, and equol, and that the lignan excretion is low. The high excretion of isoflavonoids in urine is related to the intake of soy products in the traditional Japanese diet.

We thank Anja Koskela (analytical work) and Sirkka Adlercreutz (mass spectrometry) for skillful technical assistance.

References

1. Price KR, Fenwick GR. Naturally occurring oestrogens in foods—a review. Food Addit Contam 1985;2:73–106.
2. Adlercreutz H, Fotsis T, Bannwart C, et al. Determination of urinary lignans and phytoestrogen metabolites, potential antiestrogens and anticarcinogens, in urine of women on various habitual diets. J Steroid Biochem 1986;25:791–7.
3. Setchell KDR, Adlercreutz H. Mammalian lignans and phytoestrogens. Recent studies on their formation, metabolism and biological role in health and disease. In: Rowland IR, ed. Role of the gut flora in toxicity and cancer. London: Academic Press, 1988:315–45.
4. Heller W. Flavanoid biosynthesis, an overview. In: Vody V, Middleton E Jr, Harborne JB, eds. Plant flavonoids in biology and medicine: biochemical, pharmacological, and structural-activity relationships. New York: Alan R Liss, 1986:25–42.
5. Axelson M, Sjövall J, Gustafsson BE, Setchell KDR. Origin of lignans in mammals and identification of a precursor from plants. Nature 1982;298:659–60.
6. Axelson M, Setchell KDR. The excretion of lignans in rats—evidence for an intestinal bacterial source for this new group of compounds. FEBS Lett 1981;123:337–42.
7. Setchell KDR, Lawson AM, Borriello SP, et al. Lignan formation in man-microbial involvement and possible role in cancer. Lancet 1981;2:4–7.
8. Borriello SP, Setchell KDR, Axelson M, Lawson AM. Production and metabolism of lignans by the human faecal flora. J Appl Bacteriol 1985;58:37–43.
9. Bannwart C, Adlercreutz H, Fotsis T, Wähälä K, Hase T, Brunow G. Identification of *O*-desmethylangolensin, a metabolite of daidzein, and of matairesinol, one likely precursor of the animal lignan enterolactone, in human urine. Finn Chem Lett 1984;(4–5):120–5.
10. Bannwart C, Adlercreutz H, Wähälä K, Brunow G, Hase T. Detection and identification of the plant lignans lariciresinol, isolariciresinol and secoisolariciresinol in human urine. Clin Chim Acta 1989;180: 293–302.
11. Markkanen T, Mäkinen ML, Maunuksela E, Himanen P. Podophyllotoxin lignans under experimental antiviral research. Drugs Exp Clin Res 1981;7:711–8.
12. MacRae WD, Hudson JB, Towers GHN. The antiviral action of lignans. Planta Med 1989;55:531–5.
13. Welshons WV, Murphy CS, Koch R, Calaf G, Jordan VC. Stimulation of breast cancer cells in vitro by the environmental estrogen enterolactone and phytoestrogen equol. Breast Cancer Res Treat 1987;10:169–75.
14. Adlercreutz H, Mousavi Y, Loukovaara M, Hämäläinen E. Lignans, isoflavones, sex hormone metabolism and breast cancer. In: Hochberg RB, Naftolin F, eds. The new biology of steroid hormones. Serono Symposia Publications. Vol. 74. New York: Raven Press, 1991:145–54.
15. Adlercreutz H. Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest Suppl 1990;201:3–21.
16. Adlercreutz H. Lignans and phytoestrogens. Possible preventive role in cancer. In: Rozen P, ed. Frontiers of gastrointestinal research. Vol 14. Basel, Switzerland: Karger, 1988:165–76.
17. Adlercreutz H, Höckerstedt K, Bannwart C, et al. Effect of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin. J Steroid Biochem 1987;27:1135–44.
18. Adlercreutz H, Höckerstedt K, Bannwart C, Hämäläinen E, Fotsis T, Bloigu S. Association between dietary fiber, urinary excretion of lignans and isoflavonic phytoestrogens, and plasma non-protein bound sex hormones in relation to breast cancer. In: Bresciani F, King RJB, Lippman ME, Raynaud J-P, eds. Progress in cancer research and therapy. Vol 35. Hormones and cancer 3, New York: Raven Press, 1988:409–12.
19. Armstrong BK, Brown JB, Clarke HT, et al. Diet and reproductive hormones: a study of vegetarian and nonvegetarian postmenopausal women J Natl Cancer Inst 1981;67:761–7.
20. Adlercreutz, Fotsis T, Heikkinen R, et al. Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian women and in women with breast cancer. Lancet 1982;2: 1295–9.
21. Smith RL. Recorded and expected mortality among the Japanese of the United States and Hawaii, with special reference to cancer. J Natl Cancer Inst 1956;17:459–73.
22. Nomura A, Henderson BE, Lee J. Breast cancer and diet among the Japanese in Hawaii. Am J Clin Nutr 1978;31:2020–5.
23. Dunn JE Jr. Cancer epidemiology in populations of the United States—with emphasis on Hawaii and California—and Japan. Cancer Res 1975;35:3240–5.
24. Muir C, Waterhouse J, Powell MT, Whelan S. Cancer incidence in five continents Vol 5. Lyon, France: International Agency for Research on Cancer, 1987.
25. Ota K, Misu Y. A study on latent carcinoma of the prostate in Japanese. Gann 1958;49(suppl):283–4.
26. Breslow NE, Chan CW, Dhom G, et al. Latent carcinoma of prostate at autopsy in seven areas. Int J Cancer 1977;20:680–8.
27. Yatani R, Chigusa I, Akazaki K, Stemmerman GN, Welsh RA, Correa P. Geographic pathology of latent prostatic cancer. Int J Cancer 1982;29:611–6.
28. Adlercreutz H, Honjo H, Higashi A, et al. Lignan and phytoestrogen excretion in Japanese consuming traditional diet. Scand J Clin Lab Invest Suppl 1988;48:190 (abstr).
29. Science and Technology Agency. Standard tables of food composition in Japan. 4th revised ed. Tokyo: Ministry of Finance Printing Bureau, 1982 (in Japanese).
30. Innami S, ed. Food composition tables of dietary fibers, minerals, cholesterol, fatty acids. 1st ed. Tokyo: Ishiyaku Publishing, 1985.
31. Kimura S, Yokomukai Y, Komai M. Salt consumption and nutritional state especially dietary protein level. Am J Clin Nutr 1987;45: 1271–6.
32. Fotsis T, Adlercreutz H. The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC-MS-I. Quantitation of estrogens after initial hydrolysis of conjugates. J Steroid Biochem 1987;28:203–13.
33. Wähälä K, Brunow G, Hase TA, Bannwart C, Adlercreutz H. Synthesis of deuterium labelled ethoxyamine for derivatization of estrogens as stable-isotope internal standards in GC/MS-SIM determination. Finn Chem Lett 1987;14:198–201.
34. Bannwart C, Adlercreutz H, Wähälä K, Brunow G, Hase T. Deuterium labelled ethoximes as stable isotope internal standards in the GC/MS-SIM determination of oxo-steroids in human urine extracts: preliminary results. In: Görög S, ed. Advances in steroid analysis '87. Budapest: Akadémiai Kiadó, 1988:283–6.
35. Wähälä K, Mäkelä T, Bäckström R, Brunow G, Hase T. Synthesis of the [2H]-labelled urinary lignans, enterolactone and enterodiol,

and the phytoestrogens daidzein and its metabolites equol and *O*-desmethylangolensin. J Chem Soc [Perkin 1] 1986;1:95–8.
36. Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Brunow G, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. Clin Chim Acta 1991;199:263–78.
37. Chiba K, Miyasaka M, Koizumi A, Kumai M, Watanabe T, Ikeda M. Comparison of food constituents in the diet of female agricultural workers in Japan with high and low concentrations of high density lipoprotein in their sera. J Epidemiol Community Health 1985;39: 259–62.
38. Goldin BR, Adlercreutz H, Gorbach SL, et al. The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women. Am J Clin Nutr 1986;44:945–53.
39. Goldin BR, Adlercreutz H, Gorbach SL, et al. Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women. N Engl J Med 1982;307:1542–7.
40. Kuratsune M, Honda T, Englyst HN, Cummings JH. Dietary fiber in the Japanese diet. In: Hayashi, et al, eds. Diet, nutrition and cancer. Tokyo: Japan Scientific Society Press, 1986:247–53.
41. Yokotsuka T. Soy sauce biochemistry. Adv Food Res 1986;30:195–329.
42. Adlercreutz H, Fotsis T, Höckerstedt E, et al. Diet and urinary estrogen profile in premenopausal omnivorous and vegetarian women and in premenopausal women with breast cancer. J Steroid Biochem 1989;34:527–30.
43. Adlercreutz H, Hämäläinen E, Gorbach SL, Goldin BR, Woods MN, Dwyer JT. Diet and plasma androgens in postmenopausal vegetarian and omnivorous women and postmenopausal women with breast cancer. Am J Clin Nutr 1989;49:433–42.
44. Setchell KDR, Borriello SP, Hulme P, Axelson M. Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. Am J Clin Nutr 1984;40:569–78.
45. Walz E. Isoflavone and saponin glycosides in *Soja hispida*. Justus Liebigs Ann Chem 1931;498:118–55 (in German).
46. Teas J. The consumption of seaweed as a protective factor in the etiology of breast cancer. Med Hypotheses 1981;7:601–3.
47. Baker ME. Origins of regulation of gene transcription by steroid, retinoid, and thyroid hormones. In: Hochberg RB, Naftolin F, eds. The new biology of steroid hormones. Serono Symposia Publications. Vol. 74. New York: Raven Press, 1991:187–202.
48. Martin PM, Horwitz K, Ryan DS, McGuire WL. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. Endocrinology 1978;103:1860–7.
49. Hirano T, Oka K, Akiba M. Antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast carcinoma cell line, ZR-75-1. Res Commun Chem Pathol Pharmacol 1989;64:69–78.
50. Akiyama T, Ishida J, Nakagawa S, et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 1987;262:5592–5.
51. Ogawara H, Akiyama T, Watanabe S, Ito N, Kobori M, Sedoa Y. Inhibition of tyrosine protein kinase activity by synthetic isoflavones and flavones. J Antibiot (Tokyo) 1989;41:340–3.
52. Teraoka H, Ohmura Y, Tsukada K. The nuclear matrix from rat liver is capable of phosphorylating exogenous tyrosine-containing substrates. Biochem Int 1989;18:1203–10.
53. Markovits J, Linassier C, Fossé P, et al. Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer Res 1989;49:5111–7.
54. Linassier C, Pierre M, Le Peco J-B, Pierre J. Mechanisms of action in NIH-3T3 cells of genistein, an inhibitor of EGF receptor tyrosine kinase activity. Biochem Pharmacol 1990;39:187–93.
55. Dean NM, Kanemitsu M, Boynton AL. Effects of the tyrosine-kinase inhibitor genistein on DNA synthesis and phospholipid-derived second messenger generation in mouse 10T1/2 fibroblasts and rat liver T51B cells. Biochem Biophys Res Commun 1989;165:795–801.
56. Dhar A, Paul AK, Shukla SD. Platelet-activating factor stimulation of tyrosine kinase and its relationship to phospholipase C in rabbit platelets: studies with genistein and monoclonal antibody to phosphotyrosine. Mol Pharmacol 1990;37:519–25.
57. Markaverich BMN, Clark JH. Two binding sites for estradiol in rat uterine nuclei: relationship to uterotropic response. Endocrinology 1979;105:1458–62.
58. Markaverich BM, Roberts RR, Alejandro MA, Johnson GA, Middleditch BS, Clark JH. Bioflavonoid interaction with rat uterine type II binding sites and cell growth inhibition. J Steroid Biochem 1988;30: 71–8.
59. Lehner AF, Muldoon TG, Mahesh VB, Bransome ED Jr, Hendry LB. Initial studies of a phytoestrogen-deoxyribonucleic acid interaction. Mol Endocrinol 1987;1:377–87.
60. Moore JW, Clark GM, Takatani O, Wakabayashi Y, Hayward JL, Bulbrook RD. Distribution of 17β-estradiol in the sera of normal British and Japanese women. J Natl Cancer Inst 1983;71:749–54.
61. Takatani O, Kosano H, Okumoto T, Akamatsu K, Tamakuma S, Hiraide H. Distribution of estradiol and percentage of free testosterone in sera of Japanese women: preoperative breast cancer patients and normal controls. J Natl Cancer Inst 1987;79:1199–204.
62. Baggott JE, Ha T, Vaughn WH, Juliana MM, Hardin JM, Grubbs CJ. Effect of miso (Japanese soybean paste) and NaCl on DMBA-induced rat mammary tumors. Nutr Cancer 1990;14:103–9.
63. Barnes S, Grubbs C, Setchell KDR. Chemoprevention by powdered soybean chips (PSC) of mammary tumors in rats. Breast Cancer Res Treat 1988;12:128 (abstr).
64. Troll W, Wiesner R, Shellabarger CJ, Holtzman S, Stone JP. Soybean diet lowers breast tumor incidence in irradiated rats. Carcinogenesis 1980;1:469–72.
65. Ward-Hinds M, Kolonel LN, Nomura AMY, Lee J. Stage-specific breast-cancer incidence rates by age among Japanese and Caucasian women in Hawaii 1960–1979. Br J Cancer 1982;45:118–23.
66. Adlercreutz H. The significance of intestinal microflora and diet for the metabolism and production of hormones with special reference to cancer. Fin Lakaresallsk Handl 1985;129:217–25 (in Swedish).
67. Mills PK, Beeson WL, Phillips RL, Fraser GE. Cohort study of diet, lifestyle and prostate cancer in Adventist men. Cancer 1989;64:598–604.
68. Severson RK, Nomura AMY, Grove JS, Stemmerman GN. A prospective study of demographics and prostate cancer among men of Japanese ancestry in Hawaii. Cancer Res 1989;49:1857–60.
69. Mäkelä S, Pylkkänen L, Santti R, Adlercreutz H. Role of plant estrogen and estrogen-related altered growth of the mouse prostate. In: Institute of Technology. Effects of food on the immune and hormonal systems. Schwerzenbach, Switzerland: Swiss Federal Institute of Technology and University of Zurich, 1991:135–9.

APPENDIX A

Additional experiments with a modification of the method

The method used in this study was modified further by including the determination of the plant lignan matairesinol [(3R-*trans*)-dihydro-3,4-*bis*[(4-hydroxy-3-methoxy-phenyl)methyl]-2(3H)-furanone)] (intraassay CV = 15.2% and interassay CV = 13.9%) and the isoflavonoid genistein (4',5,7-trihydroxyisoflavane) (intraassay CV = 4.5% and interassay CV = 11.6%) in the assay (1). Because further samples from the present study were not available and because of the recent great interest in genistein we used this new assay in nine other Japanese subjects (three men, three women, and three children) living in Kyoto and consuming a traditional Japanese diet before and during the 24-h urine collection.

TABLE 1A
Urinary excretion of lignans and isoflavonoid phytoestrogens (μmol/d) in nine Japanese subjects (six adults, three children) living in Kyoto and consuming traditional Japanese diet during the urine collection period

Subject, sex, age	Matairesinol	Enterolactone	Enterodiol	Total lignans	Daidzein	Equol	*O*-Desmethylangolensin	Genistein	Total isoflavonoids	Total diphenols
1, M, 41 y	0.010	0.05	0.09	0.15	5.25	6.15	0.12	15.52	27.04	27.20
2, F, 33 y	0.003	2.44	0.15	2.59	3.11	0.01	0.98	4.48	8.58	11.17
3, M, 7 y	0.003	0.07	0.09	0.16	3.23	0.01	0.06	5.66	8.97	9.13
4, M, 6 y	0.006	2.24	0.68	2.93	2.15	0.85	0.51	3.41	6.93	9.85
5, M, 8 y	0.007	0.04	3.39	3.43	3.02	0.02	0.81	4.80	8.64	12.07
6, F, 42 y	0.006	3.25	0.25	3.50	2.20	0.16	1.17	3.55	7.07	10.58
7, M, 38 y	0.012	0.70	0.25	0.96	1.60	0.07	0.40	4.93	6.99	7.95
8, M, 26 y	0.019	1.94	0.18	2.13	3.38	9.16	0.23	7.99	20.76	22.89
9, F, 30 y	0.005	0.62	0.25	0.88	1.25	3.28	0.21	1.85	6.60	7.47
$\bar{x}$	0.010	1.26	0.59	1.86	2.8	2.19	0.50	5.80	11.29	13.15
Geometric $\bar{x}$	0.010	0.50	0.27	1.17	2.58	0.25	0.35	4.91	9.81	11.89

Table 1A shows the individual urinary lignan and isoflavonoid excretion in the additional three men, three women, and three children studied by the new modified procedure, including the results of assays for matairesinol and genistein.

Reference

1. Adlercreutz H, Fotsis T, Bannwart C, Wähälä K, Brunow G, Hase T. Isotope dilution gas chromatographic-mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. Clin Chim Acta 1991;199:263–78.

D11

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 135 172
A2

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: 84109990.6

(22) Date of filing: 22.08.84

(51) Int. Cl.⁴: A 61 K 31/35
//C07D311/36

(30) Priority: 24.08.83 JP 155603/83

(43) Date of publication of application:
27.03.85 Bulletin 85/13

(84) Designated Contracting States:
BE CH DE FR GB LI NL SE

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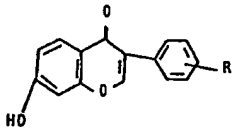
(54) **Method for treatment of osteoporosis.**

(57) A compound of the formula



wherein R is a hydrogen atom or a hydroxy group is effective for prevention or treatment of osteoporosis.

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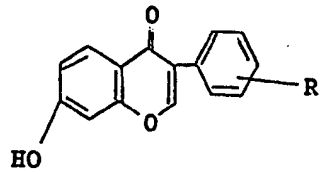
Method for treatment of osteoporosis

This invention relates to a therapeutic means for treatment of osteoporosis.

More particularly, this invention relates to a medicament for prevention or treatment of osteoporosis, which contains a compound of the formula



wherein R is a hydrogen atom or a hydroxy group.

Osteoporosis is a disease condition or illness which occurs frequently in postmenopausal females, particularly those in their sixties, and wherein the quantitative loss of bones progresses beyond a certain limit to thereby present some symptoms or risk manifestations. Among its main clinical manifestations are kyphosis, low back pain, and fractures of femoral neck, lower end of the radius, ribs, upper end of the humerus, etc. While the causative factors are variegated, including endocrine disorder and nutritional disorder, apparently the most important cause is a descreased secretion of estrogen due to hypoovarianism in females during the postmenopausal period. Therefore, of all the therapeutic agents for osteoporosis, the theoretically most effective drugs are estrogen preparations.

However, the estrogens so far available are so strong in effect as to cause side effects such as genital bleeding, mastodynia, hepatic disorder, etc. and, for this reason, have not been used recently on as many occasions as in the past. There are other types of therapeutic agents such as calcitonin, vitamin D and calcium preparations, which however are disadvantageous in that they are either only indefinitely effective or ineffective when administered by the oral route.

The present inventors have found that the compound of the formula (I) exhibits a milder estrogen activity than the conventional estrogens in the oral regimen and does not cause side effects which are produced by these known drugs but cures osteoporosis by stimulating secretion of calcitonin from the thyroid.

The compounds of the formula (I) which are employed in accordance with the present invention are invariably crystalline compounds which are white to pale yellowish brown in color, and are freely soluble in dimethylformamide and chloroform, soluble in ethanol and acetone and practically insoluble in water. When R in the formula (I) is a hydroxy group, it may be present in any position of the phenyl ring.

These compounds can be produced, for example, by cyclizing a 2,4-dihydroxy-phenyl (with or without a hydroxy group on the benzene ring) benzyl ketone to a benzopyran compound, and some of these compounds are known to have capillary vessel stabilizing activity (French Pharmaceutical Patent No. 1065), therapeutic effective for vascular disorders, inflammatory and vitamin-P deficiency disorders (United States Patent No. 3,352,754) or anticonvulsant activity (Japanese Patent Publication No. 32074/1972), but it has not been known that any of the compounds is useful for the treatment of osteoporosis.

As will be apparent from Test Example 5 which appears hereinafter, all of the compounds of the formula (I) are

sparingly toxic. Thus, in the studies in which the compounds were administered orally or subcutaneously to mice or rats at the technically feasible highest doses (5,000 to 10,000 mg/kg), there occurred no death nor toxic symptoms attributable to the compounds.

On the other hand, Test Examples 1 and 2 presented hereinafter show that 7-hydroxy-isoflavone [hereinafter referred to briefly as compound (I)] and 7,4'-dihydroxy-isoflavone [briefly, compound (II)], which are representative species of the compound represented by the formula (I), have mild estrogenic activity which is suited for the treatment of osteoporosis.

Test Example 1

Estrogenic activity of 7-hydroxy-isoflavone in young oophorectomized rats

Sprague-Dawley rats, 33 days old and 11 days after oophorectomy for elimination of endogenous estrogenic activity, were used in groups of 6 to 7 animals. Compound (I) was suspended in a 1% aqueous solution of hydroxypropylcellulose and administered orally for 3 days, while as a representative example of the conventional estrogen drug, estrone was dissolved in sesame oil and administered subcutaneously for 3 days. On the fourth day, each animal was autopsied and its uterine wet weight was recorded. As shown in Table 1, compound (I) at the daily dose levels of 200 mg/kg and 400 mg/kg produced uterine weight increasing effect with a dose-response curve of moderate gradient. In contrast, estrone showed uterine weight increasing effect with a dose-response curve of steep gradient.

Table 1

Compound	Daily dose (mg/kg)	No. of animals	Uterine wet weight (mg ± S.D.)
Compound (I)	0 (control group)	7	35.0 ± 1.0
	6.25	7	32.8 ± 1.1
	12.5	7	33.4 ± 0.9
	25	7	35.1 ± 0.8
	50	7	35.3 ± 1.7
	100	7	35.9 ± 1.0
	200	7	57.9 ± 1.0*
	400	6	70.4 ± 6.7*
Estrone	0.0025	7	101.7 ± 4.6*
	0.005	7	159.8 ± 9.4*
	0.01	7	223.3 ± 12.5*

*: Significant as compared with control group ($P<0.01$)

Test Example 2

Estrogenic activity of 7,4'-dihydroxy-isoflavone in young oophorectomized rats

Sprague-Dawley rats, 33 days old and 11 days after oophorectomy for elimination of endogenous estrogenic activity, were used in groups of 7 animals. Compound (II) was suspended in a 1% aqueous solution of hydroxypropylcellulose and administered orally. As shown in Table 2, compound (II) at the dose level of 400 mg/kg showed mild uterine weight increasing activity.

Table 2

Daily dose of compound (II) (mg/kg)	No. of animals	Uterine wet weight (mg ± S.D.)
0 (control group)	7	31.1 ± 1.1
6.25	7	33.2 ± 0.8
25	7	32.8 ± 1.0
100	7	35.3 ± 1.3
400	7	62.3 ± 6.0*

*: Significant as compared with control group ($P<0.01$)

The following Test Examples 3 and 4 show that the compounds of this invention have bone resorption-inhibiting activity which is effective for the treatment of osteoporosis.

Test Example 3

Bone resorption inhibiting activity of 7-hydroxy-isoflavone and 7,4'-dihydroxy-isoflavone in rat fetal long bone culture.

Determination of bone resorption was performed by the method of Raisz [J. Clin. Invest. 44, 103-116 (1965)]. Thus, a Sprague-Dawley rat on the 19th day of pregnancy was subcutaneously injected with 50 μ Ci of ^{45}Ca (isotope of calcium, $CaCl_2$ solution), and was laparotomized on the following day. The embryos were aseptically taken out, the forelimbs (radius and ulna) were cut off from the trunk under a binocular dissecting microscope, and the connective tissue and cartilage were removed as much as possible to prepare bone samples. Each bone sample was preincubated at 37°C for 24 hours in 0.6 mℓ of the medium containing 2 mg/mℓ of bovine serum albumin in BGJ_b medium (Fitton-Jackson modification) [GIBCO Laboratories, Grand Island, NY 14072 U.S.A.]. Then, the sample was further incubated for 3 days in the same medium as above in which 10 μg/mℓ or 25 μg/mℓ of compound (I) or 10 μg/mℓ of compound (II) had been incorporated. Then, the radioactivity of ^{45}Ca in the medium and that of ^{45}Ca in the bone were measured and the percentage (%) of

^{45}Ca released from the bone into the medium was calculated by the following formula.

Percentage (%) of ^{45}Ca released from bone into medium

$$= \frac{\text{Count of } ^{45}Ca \text{ in medium}}{\text{Count of } ^{45}Ca \text{ in medium} + \text{Count of } ^{45}Ca \text{ in bone}} \times 100$$

As control, the bones of the embryos from the same litter were similarly incubated in the absence of compound (I) or (II) for 3 days. The mean ± standard deviation for the six bones per group are shown in Table 3. It is apparent that compounds (I) and (II) suppressed bone resorption.

Table 3

	Concentration of compound	^{45}Ca (%) released	
Control group	0	20.6 ± 3.8	19.9 ± 5.0
Test group 1	Compound (I) 10 µg/mℓ	16.5 ± 2.5*	
Test group 2	Compound (I) 25 µg/mℓ	13.5 ± 2.5*	
Test group 3	Compound (II) 10 µg/mℓ		15.9 ± 1.3**

* : A significant difference from the control group ($P<0.001$)

**: A significant difference from the control group ($P<0.002$)

Test Example 4

Inhibiting activity of 7,4'-dihydroxy-isoflavone to the bone resorption potentiating action of parathyroid hormone in rat fetal long bone culture.

The bone samples prepared in the same manner as Test Example 3 were pre-incubated for 24 hours in the same medium as that prepared in Test Example 3 which contains bovine serum album in BGJ_b medium (Fitton-Jackson modification). Then, in the concomitant presence of PTH (parathyroid hormone, a bone resorption stimulant) and compound (II), the samples

were further incubated for 3 days and the percentage of ^{45}Ca released into the medium was calculated by means of the same formula as that in Test Example 3. The results are shown in Table 4. As control experiments, the same determination was made for a control group using the medium supplemented with PTH alone. It is apparent from Table 4 that compound (II) suppressed PTH-stimulated bone resorption.

Table 4

	Concentration of compound (II)	^{45}Ca (%) released
Control group	0	30.8±4.3
Test group	10 µg/mℓ	23.5±3.4*

*: A significant difference from the control group ($P<0.01$)

Test Example 5

Acute toxicity

Five-week-old ICR mice and 5-week-old Sprague-Dawley rats were respectively used in groups of 10 males and 10 females, and suspensions of compound (I) or compound (II) in olive oil were administered orally [2,500, 5,000 and 10,000 mg/kg of each compound] or subcutaneously [1,250, 2,500 and 5,000 mg/kg]. The animals were kept under observation for 14 days. None of the groups showed deaths nor toxic symptoms attributable to compound (I) or (II) and, therefore, LD_{50} values could not be calculated.

The daily dosage of the compound of the formula (I) according to this invention for human beings is generally about 1 to 50 mg/kg and preferably about 5 to 20 mg/kg for oral administration, and about 200 to 600 mg can be orally taken daily, once a day or, if necessary, in 2 to 3 divided doses. The compounds are preferably formulated into such dosage forms as tablets, capsules, etc. by the established pharmaceutical procedure. Such tablets and capsules can

be prepared using suitable excipients such as lactose, starch, etc., binders such as hydroxypropylcellulose, and lubricants such as magnesium stearate. The tablets may be sugar-coated, if necessary.

The following preparation examples are given to illustrate the invention in further detail and should not be construed as limiting the scope of the invention.

Example 1 Tablets

I)	7-Hydroxy-isoflavone	200 g
II)	Lactose	15 g
III)	Starch	44 g
IV)	Carboxymethylcellulose	10 g
V)	Magnesium stearate	1 g

The above components I) through V) were admixed to prepare 1000 uncoated tablets with a diameter of 8.5 mm.

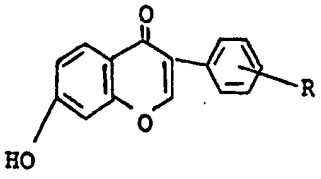
Example 2 Capsules

I)	7,4'-Dihydroxy-isoflavone	200 g
II)	Lactose	40 g
III)	Starch	50 g
IV)	Hydroxypropylcellulose	7 g
V)	Magnesium stearate	3 g

The above components I) through V) were admixed and filled into 1000 No. 1 capsules.

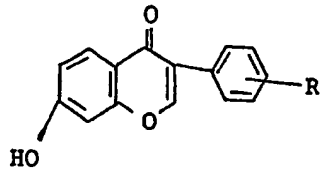
What is claimed is:

1. A compound of the formula



wherein R is a hydrogen atom or a hydroxy group for use in prevention or treatment of osteoporosis.

2. A pharmaceutical composition for prevention or treatment of osteoporosis, which contains an effective amount of a compound of the formula



wherein R is a hydrogen atom or a hydroxy group and a pharmaceutical acceptable carrier, vehicle, lubricant or diluent therefor.

3. A pharmaceutical composition according to claim 2, which is in the form of tablet, capsule, granule, fine granule, powder or syrup.

4 A pharmaceutical composition according to claim 2, wherein the osteoporosis is that caused by decreasing secretion of estrogen due to hypoovarianism.

J. Agric. Food Chem. 1983, 31, 394–396

D12

Soybean Isoflavones: Effect of Environment and Variety on Composition

Arthur C. Eldridge* and William F. Kwolek[1]

The effects of environment and variety on the isoflavones and isoflavone glucosides of soybeans were studied. Extracting the oil from soybeans did not remove the isoflavones or the isoflavone glucosides since they are not soluble in hexane. The total isoflavone of soybeans varied from 116 to 309 mg/g within variety and varied from 46 to 195 mg/g with the same variety grown in different locations. The isoflavones in soybeans also varied from year to year when soybeans were grown in the same location. On an equal weight basis most of the isoflavones are concentrated in the hypocotyl and the isoflavone content of soybean hulls is quite low.

Soybeans contain several biologically active components (Anderson et al., 1979). The isoflavones of soybean products are of interest because of their estrogenic (Drane et al., 1980; Kitts et al., 1980), antifungal (Wyman and Van Etten, 1978), and antibacterial (Naim et al., 1974) activities. The estrogenic activity of soybeans is of particular interest because soybean protein products are being used in food products such as infant formulas, health foods, and mass feeding programs.

Recently, Murphy (1981) reported the separation of two soybean isoflavone glucosides and their aglucons by gradient high-performance liquid chromatography with a methanol–water gradient. Earlier reports from this laboratory described a method for the quantitative determination of six isoflavones (daidzin, glycitin, genistin, daidzein, glycitein, and genistein) in soybeans (Eldridge, 1982a) and analysis of several commercial soybean protein products which are used for food products (Eldridge, 1982b). In the latter publication, variation was noted in the isoflavone content of several defatted soybean flours. To better understand the observed variation, we conducted the current study on the effects of variety, location, and crop year on the amount of isoflavone and isoflavone glucosides in soybeans. Distribution of the isoflavones in various seed components was also examined.

MATERIALS AND METHODS

Sources of Soybeans. All soybeans used in this study were certified seed grade. All samples except Tiger variety were supplied by Dr. Richard L. Bernard, U.S. Department of Agriculture, U.S. Regional Soybean Laboratory, University of Illinois, Urbana, IL 61801. The soybeans from the U.S. Regional Soybean Laboratory were used because as part of the soybean breeding program the varieties are grown several years at different locations. The parentage of each variety from the U.S. Regional Soybean Laboratory are included as footnotes in the tables. Tiger soybeans (TS-280) were purchased from Sommer Bros. Seed Co., Pekin, IL 61554.

Sample Preparation. For initial studies, soybeans were cracked, dehulled, flaked, and extracted with pentane–hexane (Eldridge et al., 1971). The air-dried defatted soybean flakes were analyzed and compared to full-fat (unextracted) soybean flakes.

Table I. Effect of Defatting Soybeans on Isoflavone Content of Soybean Meal[a] (mg/100 g)

isoflavone	full-fat flakes (fat-free basis)	defatted flakes	LSD[b]
daidzin	118.5	114.0	5.2
glycitin 7-β-glucoside	0.9	0.8	2.8
genistin	204.1	188.5	9.6
daidzein	2.0	2.5	1.8
glycitein	1.0	1.2	1.6
genistein	4.4	4.4	0.9
total	330.9	311.4	17.2

[a] Tiger variety with 21.4% oil. [b] Least significant difference at 0.05 probability level.

Two varieties of soybeans were cracked mechanically between rollers and hand separated into various seed parts. The hull, hypocotyl, and cotyledon were analyzed for isoflavones and isoflavone glucosides to determine the within-seed distribution.

After these initial studies, whole soybeans which were stored under identical conditions and contained 9.2–12.2% moisture were ground for 1 min in a Varco electric grinder, Model 228.1.00. The full-fat powder from this grinder gave particles, 90% of which passed a 40-mesh screen. The larger particles consisted primarily of hull fragments. At least two 1-g subsamples were taken from each soybean sample preparation.

Preparation of Extracts. A 1-g subsample in 25 mL of 80% aqueous methanol containing an internal standard (*n*-butyrophenone) was heated (boiling) on a steam bath 4 h, cooled, filtered through a type AP prefilter followed by a type HA, 0.45-μm filter (Millipore Corp., Bedford, MA).

Chromatography. The previously published high-performance liquid chromatographic procedure (Eldridge, 1982a) was followed, using a linear gradient from 25 to 50% methanol in 20 min followed by an isocratic hold period of at least 30 min. Response factors which were used for quantification of the individual isolated glucosides and aglucons were determined relative to the internal standard. These response factors were used to calculate the isoflavone and isoflavone glucoside composition of various soybean preparations.

Statistical Analysis. Variation among sample means and interactions were examined by use of analyses of variance (Snedecor and Cochran, 1980). The between-subsample variance was used to determine an LSD (least significant difference, 0.05 level) for comparing sample

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Table II. Anatomical Distribution[a] of Soybean Isoflavones in Two Varieties of Soybeans (mg/100 g)

isoflavone	hull		hypocotyl		cotyledon		
	Amsoy[b]	Tiger	Amsoy	Tiger	Amsoy	Tiger	LSD[c]
daidzin	6.6	8.6	1031.5	759.9	37.5	102.8	539.2
glycitin 7-β-glucoside			664.1	588.9	1.7	1.6	127.0
genistin	2.8	7.4	5.3	9.1	113.9	205.8	167.4
daidzein	0.7	1.0	19.0	14.0	1.4	2.8	10.6
glycitein		1.5	11.8	9.3			5.8
genistein	0.5	1.5	24.7	24.2	2.8	5.9	5.8
total	10.6	20.0	1756.5	1405.2	158.5	319.2	808.1

[a] Soybeans contain about 8% hull, 2% hypocotyl, and 90% cotyledon (Bailey et al., 1935). [b] Amsoy variety = Adams x Harosoy. [c] Least significant difference at 0.05 probability level, based on part-variety interaction.

Table III. Isoflavone Content of Different Varieties of Soybeans Grown in Urbana, IL, in 1980 (mg/100 g)

isoflavone	variety[a]				
	Hardin	Amcor	Sprite	Century	LSD[b]
daidzin	30.5	38.8	83.7	84.9	4.3
glycitin 7-β-glucoside	9.5	9.9	15.2	15.1	2.3
genistin	68.0	96.3	200.5	140.5	7.0
daidzein	2.0	0.8	3.2	3.0	1.5
glycitein	2.2	1.3	3.0	2.2	1.3
genistein	3.2	2.5	3.7	4.6	0.7
total	115.9	149.8	309.3	250.2	14.7

[a] Parentage of varieties: Hardin = Corsoy[3] x Cutlass-71; Amcor = Amsoy-71 x Corsoy; Sprite = Williams x Ransom; Century = Catlin x Bonus. [b] Least significant difference at 0.05 probability level, based on sample variation.

means for varietal and year comparisons. Interactions of variety and seed part or location were also used to estimate variability in determining LSD's. Very likely with replicated experiments the appropriate variation estimates would fall between these extremes.

RESULTS AND DISCUSSION

Defatting full-fat soybean flakes does not remove any of the isoflavones or isoflavone glucosides from soybean meal (Table I). If the values for the isoflavones in full-fat meal are corrected for the amount of oil removed by hexane (21.4%), the resulting values on a fat-free basis compare very favorably with values obtained when defatted soybean flakes are analyzed. The values in Table I agree with previously reported values for defatted soybean flours (Eldridge, 1982b). The results indicate a difference between full-fat (calculated to a fat-free basis) and defatted meals for total isoflavone and genistin content. Earlier research (Booth et al., 1960) showed that refined vegetable oils contained estrogenic compounds. Perhaps some of the free aglucons are extracted into the hexane–oil mixture. Attempts to extract the aglucons from crude soybean oil with aqueous alcohol failed to reveal any isoflavones when extracts were chromatographed. The results indicate that, in commercial practices, defatted soybean meals will contain essentially all of the isoflavones or isoflavone glucosides present in the starting soybeans.

Soybeans were cracked and separated into their anatomical parts by hand. Analyses of the hull, hypocotyl, and cotyledon for isoflavones are given in Table II. The concentration of the isoflavones on a weight basis is highest in the hypocotyl (1400–1750 mg/100 g) and lowest in the hull of the seed (10–20 mg/100 g). This is in contrast to the distribution of coumesterol in soybeans (Lookhart et al., 1979), which is concentrated in the hull or seed coat. The data in Table II also show that the hypocotyl contains ~1.5% total isoflavone and isoflavone glucoside. Why these compounds are present in the hypocotyl in such high concentrations is intriguing. The cotyledon, on the other hand, has about 20% the amount of isoflavone and isoflavone glucoside found in the hypocotyl.

The distributions of individual isoflavones are also different in the hypocotyl and cotyledon. In the hypocotyl, primarily two glucosides are found, daidzin and glycitin 7-β-glucoside, whereas in the cotyledon, there is about 20 times as much genistin as in the hypocotyl.

The isoflavone and isoflavone glucosides determined in the cotyledon are in close agreement with values found for full-fat flakes in Table I, as would be expected, because the cotyledon represents about 90% of the total seed (Bailey et al., 1935). Statistical interactions of seed part and variety were significant for daidzin, genistin, glycitein, and the total. The analytical results identified showed considerable variability between samples.

The data in Table II indicate that dehulling cracked soybeans should have little effect on the concentration of the isoflavones or isoflavone glucosides in soybean products currently produced. The data in Table II also indicate a varietal difference ($p < 0.5$) in the amount of isoflavones or isoflavone glucosides; therefore, it was of interest to study the isoflavone content of several different varieties of soybeans. Seeds were obtained, ground in a Varco mill, and analyzed without defatting. Shown in Table III is the isoflavone and isoflavone glucoside contents of four varieties of soybeans grown in Urbana, IL, in 1980. The concentrations of the isoflavones of Hardin and Amcor varieties are very similar. Century and Sprite appear to contain similar concentrations of isoflavones but have

Table IV. Isoflavone Content of Hardin and Corsoy-79 Soybeans[a] Grown in Different Locations in 1980 (mg/100 g)

isoflavone	Girard, IL		Urbana, IL		Pontiac, IL		DeKalb, IL		
	Hardin	Corsoy-79	Hardin	Corsoy-79	Hardin	Corsoy-79	Hardin	Corsoy-79	[illegible]
daidzin	14.2	25.1	22.3	49.1	15.4	64.1	44.4	53.3	25.3
glycitin 7-β-glucoside	9.2	12.2	7.4	13.0	13.1	13.3	16.1	19.0	7.1
genistin	21.5	39.5	49.9	58.5	96.3	115.6	107.5	113.9	42.7
daidzein	1.1	2.4	0.5	3.0	2.0	1.9	2.1	3.5	3.5
glycitein									
genistein	0.9	0.8	0.06	0.6	0.03	0.4	0.06	1.0	0.8
total	46.9	79.9	81.7	154.5	156.1	195.1	170.8	190.9	71.7

[a] Parentage of varieties: Hardin = Corsoy[3] x Cutlass-71; Corsoy-79 = Corsoy[4] x Lee-68. [b] Least significant difference at 0.05 probability level, based on location-variety interaction.

Table V. Isoflavone Content of Clark[a] Soybeans Grown in Urbana, IL, in Different Years (mg/100 g)

isoflavone	year 1975	1976	1978	1979	LSD[b]
daidzin	75.4	124.4	98.7	82.6	4.3
glycitin 7-β-glucoside	19.9	22.8	25.5	23.5	2.3
genistin	153.2	210.4	157.4	135.4	7.8
daidzein	2.3	0.8	1.1	1.2	1.5
glycitein	3.0	3.2	1.9	2.0	1.3
genistein	0.7	0.9	0.2	0.6	0.7
total	254.7	362.5	284.9	245.2	14.7

[a] Clark variety = Lincoln[1] x Richland. [b] Least significant difference at 0.05 probability level, based on sample variation.

larger amounts of the isoflavones than the Hardin and Amcor, even though the four samples were grown the same year in the same location. Genistin appears to vary considerably between samples.

Further investigations into different varieties were conducted. Two varieties, Hardin and Corsoy-79, were grown the same year in four different locations in Illinois. The results of the eight samples for isoflavones and isoflavone glucosides are shown in Table IV.

The concentration of the isoflavones and isoflavone glucosides vary from variety to variety, and there are also differences when the same variety is grown in different locations. Significant variety–location interactions were observed for daidzin, glycitin 7-β-glucoside, genistin, and the total isoflavones. Varietal differences at Girard and Urbana differ from the varietal differences at Pontiac and DeKalb. These results may indicate adverse growing conditions in different locales in 1980, which was a dry year in Illinois.

Table V shows the amounts of isoflavones found in Clark soybeans when grown in Urbana, IL, in different years. Significant variation among years suggests that unknown climatic and environmental factors contribute to variation in isoflavones and isoflavone glucosides.

ACKNOWLEDGMENT

We are indebted to Dr. Richard L. Bernard, U.S. Department of Agriculture, U.S. Regional Soybean Laboratory, University of Illinois, for supplying the samples used in this study and to Donna Thomas of this Center for grinding the soybean samples.

Registry No. Daidzin, 552-66-9; glycitin, 40246-10-4; genistin, 529-59-9; daidzein, 486-66-8; glycitein, 40957-83-3; genistein, 446-72-0.

LITERATURE CITED

Anderson, R. L.; Rackis, J. J.; Tallent, W. H. In "Soy Protein and Human Nutrition"; Wilke, H. L.; Hopkins, D. T.; Waggle, D. H., Eds.; Academic Press: New York, 1979; p 209.
Bailey, L. H.; Capen, R. G.; Le Clerc, J. A. *Cereal Chem.* 1935, *12*, 441.
Booth, A. N.; Beckoff, E. M.; Kohler, G. O. *Science (Washington, D.C.)* 1960, *131*, 1807.
Drane, H. M.; Patterson, D. S. P.; Roberts, B. A.; Saba, N. *Food Cosmet. Toxicol.* 1980, *18*, 425.
Eldridge, A. C. *J. Chromatogr.* 1982a, *234*, 494.
Eldridge, A. C. *J. Agric. Food Chem.* 1982b, *30*, 353.
Eldridge, A. C.; Kalbrener, J. E.; Moser, H. A.; Honig, D. H.; Rackis, J. J.; Wolf, W. J. *Cereal Chem.* 1971, *48*, 640.
Kitts, D. D.; Kirshnamurti, C. R.; Kitts, W. D. *Can. J. Anim. Sci.* 1980, *60*, 531.
Lookhart, G. L.; Finney, V. F.; Finney, P. L. In "Liquid Chromatographic Analysis of Food and Beverages"; Charalambous, G., Ed.; Academic Press: New York, 1979; Vol. I, p 129.
Murphy, P. A. *J. Chromatogr.* 1981, *211*, 166.
Naim, M.; Gestetner, B.; Zilkah, S.; Berk, Y.; Bondi, A. *J. Agric. Food Chem.* 1974, *22*, 806.
Snedecor, G. W.; Cochran, W. G. "Statistical Methods", 7th ed.; Iowa State University Press: Ames, IA, 1980.
Wyman, J. G.; Van Etten, H. D. *Phytopathology* 1978, *68*, 583.

Received for review January 22, 1982. Accepted November 1, 1982. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Three New Ingenane Derivatives from the Latex of *Euphorbia canariensis* L.

Lee-Juian Lin and A. Douglas Kinghorn*

Three new ingenane esters, 3-*O*-acetyl-16-*O*-benzoyl-20-*O*-[(*Z*)-2-methyl-2-butenoyl]-16-hydroxyingenol (1), 3-*O*-[(*Z*)-2-methyl-2-butenoyl]-16-*O*-benzoyl-16-hydroxyingenol (2), and 3-*O*-acetyl-20-*O*-[(*Z*)-2-methyl-2-butenoyl]ingenol (3), were isolated from the latex of *Euphorbia canariensis* L. by using droplet countercurrent chromatography. The structures of these skin-irritant compounds were established through the interpretation of spectroscopic data. *E. canariensis* is sold in the United States as an ornamental plant and is currently under investigation for possible cultivation as a renewable energy source. Constituents 1–3 represent a health hazard for persons who contact the latex of this species with the skin or eyes.

Euphorbia canariensis L. (Euphorbiaceae) has recently been suggested as a candidate plant for cultivation in semiarid regions to produce fuel, since its latex is rich in isoprenoids (Calvin et al., 1982). This species, although native to the Canary Islands, is now available for purchase from nurseries in the United States as an ornamental houseplant. In previous work, *E. canariensis* latex has been shown to evoke severe skin inflammation in mice (Kinghorn and Evans, 1975) and, on repeated administration subsequent to a subcarcinogenic dose of 7,12-dimethylbenz[*a*]anthracene, has produced pronounced tumor-promoting effects on mouse skin (Roe and Peirce, 1961).

A number of constituents of *E. canariensis* latex has been investigated, including inositol and a phenol oxidase

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0021-856 :/83/1431-0395$01.5C/0

D13

IN THE MATTER of European Patent Application No. 9309679.8 in the name of Graham Edmund Kelly

STATUTORY DECLARATION

I, Graham Edmund Kelly a citizen of the Commonwealth of Australia, residing at 1/47 Coolawin Road, Northbridge, New South Wales, 2063, Australia, do hereby declare as follows:

1. I am Chief Executive Office of Novogen Research Pty Ltd and I am the inventor and applicant of this application. I hold the degrees of Bachelor of Science (Veterinary) from the University of Sydney (1968); Bachelor of Veterinary Science from the University of Science (1969), Dr of Philosophy from the University of Sydney (1972). I have worked in the field of veterinary research, and general medical research, for approximately 25 years.

2. I have been involved in research on isoflavones and their biological effects since before May 1992.

3. I have read the Examiner's report from Examiner Thalmair dated 11 August 2003. I have additionally reviewed each of documents D2, D14, D26 and D32 cited by the Examiner.

4. I attach marked Exhibit **GK1** a copy of a declaration I made in connection with prosecution of a corresponding United States application. As set out in that declaration, the compositions/medicaments referred to in the claims of this European application have been shown in patient studies to be effective in the treatment of prostate cancer, premenstrual syndrome and menopause. These were patient studies in human subjects afflicted with the disorders of prostate cancer, premenstrual syndrome and menopause.

5. I understand the Examiner has rejected the claims insofar as they relate to use of isoflavone/phyto-estrogen extracts of soy or clover for the manufacture of a medicament relating to prostate cancer, based on documents D2 and D26.

6. In my opinion a person of ordinary skill in the area of biomedical research would understand that neither D2 nor D26 suggest the claimed use in the subject European application for the treatment of prostate cancer. D2 describes genistein as an anti-cancer drug against specific tumour cell lines in mice. The cell lines used are not specified. It is a well-recognized truth in the field of cancer research that no single anti-cancer drug has pan-anticancer activity. Indeed, there is a wide range of human cancers that are well known to be relatively insensitive to drugs that have potent anti-cancer activity against specific forms of cancers. Cancers generally recognized as having such insensitivity to anti-cancer drugs include pancreatic, renal and prostatic carcinoma. This insensitivity is reflected in the lack of effective drugs available clinically to treat such cancers. That is, it cannot be implied from any study showing a particular drug as having anti-cancer activity against a particular cancer, that that same drug will necessarily be active against any or all other forms of cancer. In my opinion, a person of ordinary skill in the art would recognise that D2 does not describe genistein as being a pan-cancer treatment. For the above reasons, I believe the person of ordinary skill in the art would consider the Examiner's position to be technically incorrect.

7. Likewise, document D26 describes extracts of soy beans giving rise to a particular "isoflavone" compound which is said to have a range of activities including estrogen action, anti-oxidation action, anti-haemolytic action, antilipemic action, cholesterol-lowering action and carcinostatic action. Compounds specifically referred to are daidzein and genistein. I do not consider that a person of ordinary skill in the art would regard D26 as- teaching use of extracts of soy or clover for the manufacture of medicaments for the treatment of prostate cancer. Most of the current drugs used to treat human cancer are carcinostatic, and yet very few of them show any anti-cancer activity against human prostate cancer cells in vitro, and none of them are used clinically to treat prostate cancer because of their lack of efficacy.

8. In relation to prostate cancer, I am aware that the American Cancer Society has estimated that in 2003 there will be 220,900 new cases of prostate cancer and 28,900 people will die as a result of this cancer in the United States alone. Prostate cancer is a notoriously difficult cancer to treat. The only chemotherapeutic approach of any significance is anti-androgen therapy which is designed to block the body's production of dihydrotestosterone. Drugs with classical direct cytostatic or cytotoxic effect on cancer cells are typically not used clinically because of lack of efficacy.

9. In my opinion, a person of ordinary skill in the art would readily recognise that neither D2 nor D26 teach the invention as claimed, for example in claim 1 of the subject European application insofar as it relates to prostatic cancer.

10. I believe a person of ordinary skill in the art who had documents D2 and D26 before them, and were faced with a problem of treating prostate cancer, would not be led by these references to the invention as claimed, for example in claim 1 relating to use of an isoflavone phyto-estrogen extract of soy or clover for the manufacture of a medicament for administration in unit dosage form for the treatment of prostate cancer.

11. I understand that in relation to the claims of this European application concerned with symptoms of menopause, the Examiner regards documents D14 and D32 as particularly relevant, and considers the invention is obvious in light of these documents.

12. In my opinion a person of ordinary skill in the art would regard D14 as a speculative teaching. In this regard, my corresponding US patent application was involved in an interference in the United States Patent and Trademark Office with the patent which corresponded to D14. My application in the United States prevailed in that interference, and Dr Claude Hughes, the co-author of D32, gave a declaration in those proceedings. A copy of Dr Hughes' declaration is attached in Exhibit **GK2** and is directly relevant to the teachings in D14.

13. As set out in Dr Hughes' declaration, D14 is a speculative reference, which is indefinite, uncertain and tentative (see for example paragraphs 12, 15, and 17-21 of Dr Hughes' declaration). As set out in Dr Hughes' declaration at paragraphs 23 and 24 the language of D14 is at best tentative, and speculative.

14. D26 is a review article, and as I understand the Examiner's report, the Examiner relies on the description of D32 at page 88 right hand column, paragraph 4. I have reviewed this portion of D32 and note that it is predicated upon Chinese herbal medicine. The nature of the phyto-estrogens involved in the purported effects are not described, nor is the nature of the Chinese herbs used. It must be recognised that as review, D26 describes in the right hand column of page 88 the deleterious roles of phyto-estrogens in human disease, including vascular disease, coronary heart

disease and possible cancer initiation. On balance, the summary at the conclusion of D32 mentioned at the bottom of page 88 right hand column through page 89 is that the majority of the effects of phyto-estrogens are noxious, which directs away from using phyto-estrogens in human disease.

15. I believe that a person of ordinary skill in the art faced with documents D14 and D32, and additionally faced with a problem of treating menopause symptoms, would not consider it obvious to try extracts of soy or clover for the treatment of symptoms of menopause. In part the noxious effects of phyto-estrogen compounds would teach away from such a treatment. Further, there is nothing in D14, or D32 for that matter, to suggest that phyto-estrogen extracts of soy or clover would be useful in treating menopause symptoms.

AND I MAKE this solemn declaration by virtue of the Statutory Declarations Act 1959 as amended and subject to the penalties provided by that Act for the making of false statements in statutory declarations, conscientiously believing the statements contained in this declaration to be true and correct in every particular.

_____

Graham Edmund Kelly

Before me: _____

IN THE MATTER of European Patent Application No. 9309679.8 in the name of Novogen Research Pty Ltd

This is Exhibit GK1 referred to in the Statutory Declaration of Graham Edmund Kelly made before me.

DATED this day of 2003

Before me: _____

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of	)	Examiner: J. Wilson
GRAHAM EDMUND KELLY	)	
Application No. 08/338,567	)	Group Art Unit: 1211
Filed: January 12, 1995	)	
For: HEALTH SUPPLEMENTS CONTAINING PHYTO-OESTROGENS ANALOGUES OR METABOLITES THEREOF	)	

DECLARATION OF GRAHAM EDMUND KELLY UNDER 37 C.F.R. §1.132

I, Graham Edmund Kelly, a citizen of the Commonwealth of Australia, residing at 1/47 Coolawin Road, Northbridge, New South Wales, Commonwealth of Australia, do solemnly and sincerely declare as follows:

1. I am Chief Executive Officer of Norvet Ltd. and am the inventor of the subject application.

2. I am a research scientist and hold the degrees of Bachelor of Science (Vet) from the University of Sydney (1968); Bachelor of Veterinary Science from the University of Sydney (1969); and Doctor of Philosophy from the University of Sydney (1972). I have worked in the field of medical and veterinary research for approximately twenty-five years.

3. I have read the Office Action in connection with U.S. Patent Application No. 08/338,567 by Examiner Wilson, dated 10 September 1996.

4. The health supplement composition comprising an extract from soya or clover as claimed in the patent application has been used in a series of therapeutic treatments conducted at my request and/or under my supervision. Details of these treatments are set forth below.

Compositions

Compositions comprising an extract of soya or clover were prepared in accordance with Examples 1 and 2 at pages 18 and 19 of the subject application 08/338,567. These compositions, for convenience referred to as "the inventive composition", were prepared comprising 40 mg, 80 mg, 120 mg, 160 mg and 240 mg of phyto-estrogen.

Treatments

Prostate Cancer

Two patients diagnosed with prostate cancer were treated initially with the inventive composition comprising 240 mg per day, and subsequently 120 mg per day phyto-estrogen. The PSA levels, a marker for prostate cancer, were stabilized in these patients and there has been no rise in the PSA levels subsequently. This demonstrates the treatment of prostatic cancer in these individuals.

A further patient diagnosed with malignant prostate cancer (PSA 13.1 µg/L) was treated with the inventive composition. The patient was treated with the composition comprising 160 mg per day phyto-estrogen, seven days prior to prostatectomy. Histological comparison was made of the pre-operative needle biopsy and the prostatectomy specimen. The needle biopsy revealed low grade infiltrating adenocarcinoma. The prostatectomy specimen showed mild patchy microvacuolation and prominent apoptosis (programmed cell death). Lymph nodes were negative for malignancy. The degenerative changes in the prostatectomy specimen, especially the apoptosis, show treatment of the prostatic cancer.

Benign or Cystic Breast Disease

A patient with benign or cystic breast disease was treated with 160 mg of the inventive composition administered orally on a daily basis. The patient exhibited no breast tenderness, which was maintained when the dosage level was reduced to 80 mg. Her symptoms did not return and she continues to have relief from mastalgia.

Pre-Menstrual Syndrome (PMS)

Nine woman were treated with 80 mg per day of the inventive composition and were screened for the well-described symptoms of PMS including psychological, psychiatric, gynecological and personal status. Relief from PMS in these various symptoms was observed across the treatment group.

Menopause

Eight menopausal women were divided into two groups of four and treated with either 40 mg or 160 mg of the inventive composition administered orally on a daily basis. Four patients were also treated with a placebo composition. Indicators measured were incidence or severity of hot flushes, night sweats. Green score, vaginal pH, vaginal cytology and mean cholesterol levels across the treatment groups. A significant change in menstrual symptoms was observed and a dose response change was observed between the 40 mg and 160 mg dosage range. This indicating that 160 mg per day was the most effective dosage for treatment of menopausal symptoms.

5. These studies show that a composition according to the invention described and claimed in U.S. Patent Application No. 08/338,567 is effective in the treatment of:

- Prostate cancer

- Benign or cystic breast disease (mastalgia)
- Pre-menstrual syndrome
- Symptoms of menopause

6. As shown in the Examples 3 and 4 at pages 19 and 20 of the subject application 08/338,567, a composition according to the invention was effective in the treatment of elevated levels of cholesterol in the blood stream.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further, that the statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and such willful false statements may jeopardize the validity of the application or patent issuing thereon.

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GRAHAM EDMUND KELLY

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CLIMACTERIC 1999;2:79–84

The effect of Promensil™, an isoflavone extract, on menopausal symptoms

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Key words: ISOFLAVONE EXTRACT, PROMENSIL™, FLUSHES, MENOPAUSAL SYMPTOMS

ABSTRACT

Objectives The primary aim was to assess whether the use of an isoflavone extract containing 40 mg or 160 mg of total isoflavones affects the frequency of menopausal flushes and other symptoms. The secondary aims were assessments of possible effects on menopause symptom scores and biological measures of estrogen activity.

Methods A randomized, double-blind, placebo-controlled prospective trial of 37 postmenopausal women with symptoms of estrogen deficiency was performed over a 12-week period. The women were randomized to three treatment groups: placebo, 40 mg or 160 mg, delivered in tablet form.

Results There was no significant difference in the incidence of flushes between the three groups at trial conclusion. There was no difference between the groups in Greene Menopause Symptom Scores, vaginal pH, levels of follicle stimulating hormone (FSH), sex hormone binding globulin (SHBG) or total cholesterol, liver function or blood parameters. A statistically significant increase in high-density lipoprotein (HDL) cholesterol of 18.1% ($p = 0.038$) occurred in the 40-mg group.

Conclusion A large placebo response and inadvertent use of dietary isoflavones in the placebo group may have obscured a significant change in flushing frequency. Previous uncontrolled studies claiming a beneficial effect of foods with a high isoflavone content on menopausal symptoms may have been confounded by a large placebo response.

INTRODUCTION

Menopausal hot flushes are the most common symptom of the climacteric, and occur in 60–75% of women undergoing a natural menopause with a higher incidence after surgical menopause[1]. Flushes may occur at any time, and are characterized by a flush usually starting in the face, spreading to the neck, head, chest and rest of the body, and can severely disrupt a woman's life. Flushes are often accompanied by the subjective feeling that others notice the occurrence of these symptoms. Flushes also occur at night, awaking women from sleep, with consequences including insomnia, irritability, tiredness and general lethargy. Estrogen is a very effective treatment of flushes; however, in certain clinical settings, the use of estrogen in some women is undesirable or contraindicated. Other women may have a resolution of symptoms with estrogen replacement but find the side-effects of estrogen more undesirable, even in small amounts. Despite clinical benefits attributed to the use of hormone replacement therapy (HRT) in postmenopausal women, compliance ranges from 10 to 50%[2], and alternatives to estrogen replacement would be therapeutically valuable in these cases.

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ORIGINAL ARTICLE

Received 24-08-98
Revised 3-12-98
Accepted 10-12-98

The apparent cultural differences in the incidence of menopausal flushing[3,4] have been attributed to differences in dietary intake of isoflavones, naturally occurring plant compounds with a similar spatial structure to estrogen and estrogen-like biological activity[5,6]. Much of the evidence regarding the effects of isoflavones on menopausal symptoms is epidemiological, and has been linked to the consumption of soy products in different populations. This indicates soy products to be a major source of isoflavones. Soybean and its products appear to have high levels of isoflavones, the extent depending on the type and manner of preparation[7,8]. Intervention by dietary modification, with foods containing isoflavones, has been shown to alter biochemical markers associated with reproductive function[9]. The consumption of soy products is estimated to be highest in Japanese populations, with levels in the diet of up to 200 mg per day[10]. There is a gradation in consumption of isoflavones in the diet from Asia, where consumption is estimated to be 25–45 mg of total measurable isoflavones per day, to Western countries, where less than 5 mg per day are consumed[11]. A Western-type diet elevates plasma levels of sex hormones and decreases the sex hormone binding globulin (SHBG) concentration, increasing the bioavailability of the sex steroids[12]. The same diet also results in low formation of isoflavone[12].

The hypothesis that supplementation with isoflavones has an estrogenic effect in postmenopausal women and relieves menopausal symptoms was investigated. The primary outcome measure was the frequency of hot flushes, with secondary outcomes being menopause symptom scores and biological measures of estrogen activity. Supplementation was achieved using Promensil™ (Novogen Ltd, Sydney, Australia), a tablet isoflavone supplement prepared from red clover extract.

MATERIALS AND METHODS

A double-blinded, randomized, placebo-controlled trial was performed consisting of three arms: placebo, one tablet (40 mg) of Promensil and four tablets (160 mg) of Promensil. Promensil is a standardized isoflavone supplement prepared from red clover extract, in tablet form. Each tablet contained 40 mg of total isoflavones comprising the four primary isoflavones: genistein (4.0 mg) and daidzein (3.5 mg), and their methylated precursors biochanin (24.5 mg) and formononetin (8.0 mg).

Thirty-seven subjects were recruited through the University Department of Obstetrics and Gynaecology at St. George Hospital, Sydney, Australia. The inclusion criteria for the trial were postmenopausal women who were symptomatic, having at least three flushes per day. Menopause was defined by bilateral oophorectomy or amenorrhea for at least 6 months with typical symptoms of the menopause, and a serum follicle stimulating hormone (FSH) level greater than 40 IU/l. Age was restricted to 40–65 years, upon trial entry. Participants were instructed not to alter their usual diet for the duration of the study. The exclusion criteria included HRT use within the previous 6 weeks; allergy to foodstuffs known to contain isoflavones; current history of active bowel, liver or gallbladder disease; diabetes requiring drug therapy; and malignancy (excluding skin cancers). Women with contraindications to HRT use, vegetarians and/or regular soy product users and those receiving medications that result in liver enzyme induction were also excluded.

Pre-trial flushing was assessed using a daily flush diary for the week prior to trial entry. The severity of menopausal symptoms was assessed during this period, using the Greene Menopause Scale. A 24-h urine collection for isoflavone measurement was performed during this week. Upon fulfillment of inclusion criteria, subjects were entered into the study. The randomization procedure was performed by an external statistician. The allocation schedule was produced in random permuted blocks of six, generated using a computer random number generator. Subsequently, tablets were packed in daily sachets and supplied in individual subject containers to the investigators. Packaged subject containers were received prior to trial recruitment. There was no contact between the generator of randomization and the executor of the trial, prior to, during or after the trial.

After screening, the subjects were randomly assigned to placebo or one of the active treatment groups. Physical and vaginal examinations were performed. A vaginal wall smear for determination of maturation value[13] and pH was performed at trial entry. The vaginal wall smear was performed using a speculum and wooden spatula, with a single pass along each lateral vaginal wall. Blood was collected in a non-fasting state for assessment of hematological profile, liver function and serum levels of FSH and SHBG. Hematology and biochemistry were performed by Southpath Laboratories at St. George Hospital (Sydney,

Australia), with the lipid biochemistry assayed using standard enzymatic colorimetric kits on a BM/Hitachi 747 analyzer. Levels of FSH and SHBG were assayed by the Endocrine Laboratory at the Royal Hospital for Women (Sydney, Australia). The FSH level was determined with an automated chemiluminescence system, Chiron Diagnostic ACS : 180, and the SHBG by chemiluminescent enzyme immunometric assay on an Immulite Automated Analyzer. A 24-h urine collection for isoflavone measurement preceded trial entry. Urinary isoflavone assays were performed by Novogen Ltd. Each sample was analyzed using high-performance liquid chromatography (HPLC) for identification of the isoflavonoids: genistein, daidzein, biochanin A, formononetin and equol. All analyses were run in duplicate. The reproducibility of the method has been previously reported[14]. Synthesized isoflavonoids for use as standards were obtained from the Department of Organic Chemistry, University of Helsinki.

The trial was 12 weeks in length, and the subjects were seen every 4 weeks for clinical assessment, compliance checks and assessment of flush count and Greene Score. Contact between appointments was provided if required by individual subjects. In the final week of the study, physical and vaginal examinations, the vaginal smear, and urine and blood collections were repeated. Compliance was assessed by return of tablet containers and urinary isoflavone levels. All subjects were included in the analysis on an intention-to-treat basis. On a *post hoc* basis, the stored serum was analyzed for serum total cholesterol and high density lipoprotein (HDL) cholesterol levels.

The Greene Menopause Score is a validated menopause symptom self-assessment form, and was completed weekly. For analysis, the Greene Score was calculated as a total and in the accompanying subgroups, the Psychological Scale, Somatic Scale and Vasomotor Scale. Data were entered into Statistica™ (StatSoft™ Inc, Tulsa, USA). Non-parametric data were analyzed using the Kruskal–Wallis non-parametric analysis of variance. Normally distributed data were analyzed using one-way analysis of variance. Any difference between individual groups was re-analyzed using the Newman–Keuls test, provided that the F value from the analysis of variance was significant. This test was preferred to the Bonferoni modification, a corrected Student's t test, as the Newman–Keuls is based on the least significant difference between the means of each group and is a 'protected' test, taking into account the risk of specious results arising from multiple comparisons. Data are presented in the format mean ± standard deviation with p values included when ≤ 0.05.

All subjects consumed four tablets daily. Placebo and active tablets were of the same appearance (in color and size) and taste. Each day's tablets were in an individually marked sachet, containing four placebo tablets, one active and three placebo tablets (40-mg group) or four active tablets (160-mg group). The code was broken only after trial completion, analysis of serum and urine samples, database entry with subsequent checking and locking of the database.

RESULTS

Thirty-seven subjects were randomized. One subject withdrew for personal reasons before commencing any treatment, and a further subject was recruited to the same randomization position. Twelve subjects were randomized to each of the placebo and 40-mg groups and 13 to the 160-mg group. Two patients were subsequently withdrawn from the 160-mg group because of intervention by their general practitioners. All packages containing tablets were returned. Tablets were not taken by only two patients, with days of missed tablets being less than 7.

The ages of the women participating in the study were 53.1 ± 2.5, 54.5 ± 4.4 and 56.1 ± 3.9 years in the placebo, 40-mg and 160-mg groups, respectively. The age at menopause in these groups was 47.7 ± 8.0, 48.5 ± 3.6 and 51.1 ± 8.8 years. There were no statistically significant differences in age, weight and age at menopause between the groups. Weight was monitored throughout the study and there was no difference in these values within each group, between trial entry and exit.

Flushing frequency decreased in all groups over the 12-week period of the trial. There was no difference in flushing frequency between the active and placebo groups. These data and those of other measures of estrogen activity are presented in Table 1. Analysis of urinary isoflavone levels showed a dose-dependent increase between week 0 and week 12 in groups receiving active tablets. A smaller but notable increase was also observed in the placebo group, indicating that subjects in this group may have adopted altered dietary patterns to include foods containing isoflavones. This occurred despite inclusion criteria requests that dietary patterns should not be altered throughout the course of the trial.

Table 1 Biological and biochemical markers of estrogen activity

	Trial week	Placebo	40 mg Promensil™	160 mg Promensil™
Number of flushes	0	8.6 ± 4.6	6.9 ± 2.1	9.0 ± 5.2
	12	5.8 ± 4.5 (–35%)	4.9 ± 4.8 (–29%)	5.9 ± 4.6 (–34%)
Greene Score	0	18.5 ± 11.4	19.9 ± 10.6	19.9 ± 4.4
	12	9.9 ± 5.9 (–46%)	11.2 ± 8.8 (–44%)	14.7 ± 16.8 (–26%)
FSH (IU/l)	0	75.9 ± 26.6	87.7 ± 31.6	69.9 ± 24.9
	12	76.2 ± 29.4	82.2 ± 30.2	58.7 ± 22.3
SHBG (IU/l)	0	66.2 ± 46.0	72.6 ± 28.2	66.7 ± 28.7
	12	64.7 ± 62.2	64.1 ± 26.0	55.8 ± 19.1
Maturation value	0	51.3 ± 1.7	49.4 ± 2.2	51.1 ± 1.7
	12	49.9 ± 7.8	45.8 ± 24.1	51.1 ± 1.9
Vaginal pH	0	5.3 ± 0.8	5.4 ± 0.7	5.1 ± 0.6
	12	5.4 ± 0.7	5.1 ± 0.9	5.0 ± 0.8
Urinary isoflavone (ng/ml)	0	2.68 ± 1.92	3.43 ± 2.07	3.70 ± 2.96
	12	3.67 ± 2.79	9.40 ± 5.67	28.18 ± 17.52
HDL cholesterol (mmol/l)	0	1.08 ± 0.31	1.05 ± 0.40	1.06 ± 0.80
	12	1.13 ± 0.28	1.24 ± 0.49*	1.19 ± 0.40

*p = 0.038, compared to week 0; FSH, follicle stimulating hormone; SHBG, sex hormone binding globulin; HDL, high-density lipoprotein

None of the biological parameters of estrogen activity measured, including FSH, SHBG and climacteric symptom scores, showed any change with time, compared to placebo. Analysis of the vaginal smears showed no difference between the groups in vaginal maturation value, although 20% of the smears were unable to be evaluated owing to severe atrophy. There was no increase in vaginal acidity associated with isoflavone use (Table 1).

Serum HDL cholesterol levels increased significantly by 18% (p = 0.038) in subjects taking the 40-mg dose of isoflavones. The results for 160 mg did not differ from those for placebo. Total cholesterol and triglyceride levels could not be accurately evaluated as the samples were collected in a non-fasting state.

DISCUSSION

The paucity of published data concerning the effects of isoflavones as an intervention in the treatment of menopausal symptoms, in the presence of epidemiological data suggesting that these compounds may modify many physiological processes including the menopause, formed the basis of the present study. A recent larger study has suggested that isoflavones from soy protein may modify flushing frequency in women with severe flushing to a moderate degree (45% reduction in flushes compared to 30% reduction in the placebo group)[13].

A similar pattern was observed in the trial reported here. An overall decrease in flushing frequency in the treatment groups was matched by a large fall also in the control group. The data from this study suggested that a trend towards improvement on active medication may have been obscured by the large placebo effect and the highly variable responses to treatment apparent for both the placebo and active treatments. The decrease in flushing frequency in the placebo group should be interpreted with regard to the increase in urinary isoflavone excretion in this group at trial exit. These effects may also be consistent with the data from small intervention trials already published and represent placebo response and time effects[16].

Closer scrutiny of individual data revealed other difficulties within the control group that may have altered the final analysis. Two subjects were suspected by the investigators of changing the criteria on which flushing frequency was based when completing the diary cards, after enrollment into the trial. Both subjects initiated lengthy discussions at the enrollment visit, and after completion of the weekly pre-trial flush count. Information and education of these patients appear to have altered their perception of determining or characterizing hot flushes. In these two patients, there was a fall in flushing frequency within a week of enrollment from more than ten flushes daily to one or two each day. Another in the placebo group grew alfalfa in her garden and consumed this while taking part in the trial, inadvertently increasing her isoflavone

exposure. This was reflected by a rise in the urinary isoflavone levels for this patient. The increased overall exposure to isoflavones noted in the urinary levels of the control group may have contributed to the placebo response reported. However, the study was performed on an intention-to-treat basis and, therefore, all subjects were included in the analysis.

No differences between the control and the active groups were observed in the subjective scoring of menopausal symptoms. The Greene Climacteric Scale has been previously validated as an assessment of menopausal symptoms. The Vasomotor Symptom Score assesses severity of vasomotor function as a numerical measure of occurrence; however, it does not address subjective assessment of the intensity of flushes or night sweats. Lack of effect is certainly an explanation, and inability to detect an effect is another. In addressing this issue, it is appropriate that questionnaires used for subjective assessment of menopausal symptoms in future studies are capable of detecting changes in the intensity of symptoms as well as the frequency.

While urinary levels of isoflavones increased in the active groups, there were subjects in both the 40- and the 160-mg groups who had a fall in urinary isoflavone levels, compared to trial entry. Return of packaging suggested good compliance, and, while falls in the total urinary isoflavones with time may indicate poor compliance, they may also be indicative of individual variations in absorption profile. There are minimal pharmacokinetic data available for isolated isoflavone use in humans; however, preliminary data suggest individual differences in the metabolism of daidzein to its end-metabolites, equol and *O*-DMA[17]. Recent data suggest that isoflavones are readily absorbed via the gut, and both daidzein and genistein serum levels reflect, in a positive relationship, the oral dose consumed[18]. Of note in this recent trial is that equol was rarely detected in the urine of the study patients while *O*-desmethyl-angolensin levels appeared to rise in these groups.

The present study confirms the results of others, in that isoflavones have no effect on serum levels of FSH and SHBG. The lack of change from baseline and between groups in hematological parameters, liver enzymes and proteins suggests that the doses used in the study cause no gross abnormalities in hematological and liver function in the short or medium term. Debate has also ensued over isoflavone effects on vaginal epithelium and pH, used as biological indicators of estrogen activity. The present data suggest that isoflavones have no effect on vaginal epithelium or vaginal pH.

The hypocholesterolemic effects of dietary soy protein have been previously demonstrated. In a meta-analysis of dietary soy protein and the effects on serum lipids, Anderson and colleagues showed a statistically significant decrease in total cholesterol and low-density lipoprotein (LDL) cholesterol levels in hypercholesterolemic subjects[19]. In this analysis, there was a non-significant trend towards a rise in HDL cholesterol. The authors suggested that 60–70% of the effects on serum lipid levels by soy protein are as a result of the presence of isoflavones in the soy protein. As the lipid levels were assessed on a *post hoc* basis in the present study and were collected in the non-fasting state, LDL cholesterol levels were not determined. The rise in HDL cholesterol of 18.1% seen in the 40-mg Promensil group may represent a window effect. There may be a response to low levels of isoflavones, with diminution or loss of the effect as serum levels increase.

The present study emphasizes many of the problems with pharmaceutical-style intervention studies using naturally occurring dietary compounds. There is a need for further larger studies investigating the areas of clinical effectiveness of isoflavone supplementation in the treatment of menopausal symptoms. Initial indications of biological activity require appropriate assessment of pharmacokinetic properties and dose–response relationships. Although these compounds may well play a complementary role in the treatment of menopausal symptoms and disease associated with estrogen deficiency, issues concerning dose–response relationships, therapeutic variability and side-effect profiles at these doses, and effects on long-term diseases associated with estrogen deficiency, remain to be addressed.

ACKNOWLEDGEMENTS

The authors would like to thank Professor L. Howes for his advice upon review of this article.

Conflict of Interest J.A.E. is a consultant for Novogen Ltd. J.B.H. is currently employed by Novogen Ltd as a research scientist but was not employed by the company at the time of the study. D.C.K. nil.

Source of funding This study was sponsored by Novogen Ltd, Sydney, Australia.

References

1. Sturdee D, Brincat M. The hot flush. In Studd JWW, Whitehead MI, eds. *The Menopause*. Oxford: Blackwell Scientific Publications, 1988: 24–42
2. Hahn RG. Compliance considerations with estrogen replacement: withdrawal bleeding and other factors. *Am J Obstet Gynecol* 1989;161: 1854–8
3. Boulet MJ, Oddens BJ, Lehert P, Vemer HM, Visser A . Climacteric and menopause in seven south-east Asian countries. *Maturitas* 1994;19: 157–76
4. McCarthy T. The prevalence of symptoms in menopausal women in the Far East: Singapore segment. *Maturitas* 1994;19:199–204
5. Aldercreutz H, Hamalainen E, Gorbach S, Goldin B. Dietary phytoestrogens and the menopause in Japan [Letter]. *Lancet* 1992;339:1233
6. Knight DC, Eden JA. A review of the clinical effects of phytoestrogens. *Obstet Gynecol* 1996; 87:897–904
7. Price KR, Fenwick GR. Naturally occurring estrogens in foods – a review. *Food Addit Contam* 1985;2:73–106
8. Axelson M, Sjövall J, Gustafsson BE, Setchell KDR. Soya – a dietary source of the non-steroidal estrogen equol in man and animals. *J Endocrinol* 1984;102:49–56
9. Morton MS. Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. *J Endocrinol* 1994;142: 251–9
10. Cassidy A, Bingham S, Setchell KDR. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am J Clin Nutr* 1994;60:333–40
11. Coward L, Barnes NC, Setchell KDR, Barnes S. The isoflavones genistein and daidzein in soybean foods from American and Asian diets. *J Agric Food Chem* 1993;41:1961–7
12. Aldercreutz H. Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. *Scand J Clin Lab Invest* 1990;201:53–23
13. Koss LG. Cytological evaluation of the endocrine status of the woman. In Koss LG, ed. *Diagnostic Cytology and its Histopathological Bases*, 3rd edn. Philadelphia: JB Lippincott Co., 1979;208
14. Franke AA, Custer LJ, Cerna CM, Narala K. Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. *Proc Soc Exp Biol Med* 1995;208:18–26
15. Albertazzi P, Pansini P, Bonaccorsi G, Zanotti L, Forini E, de Aloysio D. The effect of dietary soy supplementation on hot flushes. *Obstet Gynecol* 1998;91:6–11
16. Murkies AL, Lombard C, Strauss BJG, Wilcox G, Burger HG, Morton MS. Dietary flour supplementation decreases post-menopausal hot flushes: effect of soy and wheat. *Maturitas* 1995; 21:189–95
17. Kelly GE, Joannou GE, Reeder AY, Nelson C, Waring MA. The variable metabolic response to dietary isoflavones in humans. *Proc Soc Exp Biol Med* 1995;208:40–3
18. Howes JB, Eden JA, Howes L. Acute and chronic pharmacokinetics of daidzein and genistein in man. Presented at the *St George Hospital Scientific Symposium*, Sydney, Australia, 1997: abstr
19. Anderson JW, Johnstone BM, Cook-Newell ME. Meta-analysis of the effects of soy protein intake on serum lipids. *N Engl J Med* 1995;333:276–82

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Maturitas 42 (2002) 187–193

MATURITAS
THE EUROPEAN MENOPAUSE JOURNAL

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Review

Isoflavones from red clover (Promensil®) significantly reduce menopausal hot flush symptoms compared with placebo

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Received 18 October 2001; received in revised form 9 April 2002; accepted 6 May 2002

Abstract

Objectives: To investigate the effectiveness and safety of a red clover isoflavone dietary supplement (Promensil, Novogen Ltd., Australia) versus placebo on the change in hot flush frequency in postmenopausal women. *Methods:* In this randomized, double blind, placebo-controlled trial 30 women with more than 12 months amenorrhoea and experiencing more than five flushes per day were enrolled. All received single blind placebo tablets for 4 weeks and were subsequently randomized to either placebo or 80 mg isoflavones for a further 12 weeks. Efficacy was measured by the decrease in number of hot flushes per day and changes in Greene Climacteric Scale Score. *Results:* During the first 4 weeks of placebo the frequency of hot flushes decreased by 16%. During the subsequent double blind phase, a further, statistically significant decrease of 44% was seen in isoflavones group ($P < 0.01$), whereas no further reduction occurred within the placebo group. The Greene score decreased in the active group by 13% and remained unchanged in the placebo group. *Conclusion:* In this study, treatment with 80 mg isoflavones (Promensil) per day resulted in a significant reduction in hot flushes from baseline. At the end of the study there was a significant decrease in hot flushes of 44% between the active and placebo group, demonstrating the effectiveness of Promensil in the management of hot flushes.

Keywords: Menopause; Isoflavone; Phytoestrogen; Hot flush

1. Introduction

Menopausal symptoms, especially hot flushes, have been reported to vary between countries. Postmenopausal women in Europe and North America report an incidence of hot flushes as high as 70–80%, while women in Japan, China and Southeast Asia, report the rate of 25 [1], 18 [2] and 14% [3], respectively [4]. It has been speculated that these differences may be due to estrogenic plant compounds in their diets. Traditional diets in South American, Mediterranean and Asian countries are high in many different varieties of legumes [5]. One such group of plant compounds, which are present in legumes, is the isoflavones. Isoflavones are molecules with similar spatial chemical structures to steroids. They have been shown in vitro to bind and interact with the

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0378-5122/02/$ - see front matter
PII: S0378-5122(02)00080-4

estrogen receptor (ER), predominantly the ER beta form of the receptor, and exert a weak estrogenic effect [6]. Isoflavones are now being extracted and produced as dietary supplements for a variety of indications, some of which are currently treated predominantly with steroidal estrogens. The study medication (Promensil) is a standardized extract of red clover isoflavones that has passed independent testing for label accuracy. Each tablet delivers 40 mg of formononetin, daidzein, biochanin and genistein [7] in the biologically active aglycone forms.

Randomized placebo-controlled studies are necessary for proof of any pharmacological effect. This is especially true when testing products for indications that have a potentially high placebo effect. There have been conflicting reports on the efficacy of such products for the treatment of hot flushes [8–11]. Differences could be attributed to poor study design with low patient numbers, lack of control of dietary intake of isoflavones and inclusion of patients with too few hot flushes per day at baseline. Recently studies have adopted more stringent selection criteria [12,13], such as more than five hot flushes per day at randomization, a criterion that has also been used in hormone replacement therapy (HRT) studies [14].

This study was designed to investigate the efficacy in reduction of the number of hot flushes in Dutch postmenopausal women with a standardized isoflavone preparation. Hot flushes are the principal menopausal symptom of concern in Dutch postmenopausal women [15]. It has also been established that in The Netherlands the diet is generally low in legumes [16], which may make it easier to control the dietary isoflavone intake of the participants.

2. Methods

2.1. Study design

This study was double blind, randomized placebo-controlled trial with a single blind, 4 weeks, placebo screening phase. The local Ethics Committee approved the protocol and participants gave informed consent before the start. Participants were given a list of 'foods to avoid', which included legumes and isoflavone supplements. They were instructed to record the number of hot flushes each day throughout the study on a diary and to score a list of 21 symptoms [17] as non-existent, mild, moderate or severe. The baseline hot flush count was calculated as the average count of the last 7 days from the 4 weeks screening phase. Only the number of hot flushes were used as an inclusion criteria, not the severity of hot flushes. Women with an average of more than five hot flushes per day were then randomized to either two tablets (Promensil, 40 mg) or two placebo tablets, and were instructed to take these tablets every morning for the entire 12 weeks. Both types of tablets (active and placebo) were in identical in size, color, and weight. During the 4 weeks run-in phase only placebo tablets were used (single blinded).

The isoflavones in the active tablets were manufactured from three varieties of red clover using a standardized extraction and blending process to obtain a proprietary ratio of daidzein, genistein, biochanin and formononetin. A 24 h urine specimen was collected at screening (visit 1), before randomization (visit 2) and at study end (visit 3). All study medication for the second phase of the study was numbered by the Hospital Pharmacy. Each batch number was put in a blank sealed envelope. All envelopes were shuffled and then again numbered from 1 to 36 on the outside and handed over to the study nurse who gave out envelopes in successive numbers. Participants took their envelope to the pharmacy where the number in the envelope was matched with the batch number on the medication. Inclusion would stop at either 30 participants included or after 6 months of active screening. In total 30 numbers were given out after 6 months screening.

2.2. Study participants

Thirty symptomatic postmenopausal women, aged 49–65 years were recruited into the study. Postmenopause was defined as at least 12 months amenorrhoea. Women were excluded if they received HRT or antibiotics within 12 weeks of study entry, had undiagnosed vaginal bleeding,

active liver or renal disease, a history of allergy for foodstuffs or a previous history of malignancy, cardiovascular disease or thromboembolism.

2.3. Urinary isoflavone analysis

The total volume of the 24 h specimen was measured and recorded before a 100 ml aliquot was collected and stored at −8 °C. The frozen samples were shipped on dry ice and assayed at Novogen Laboratories (North Ryde, Australia) using high performance liquid chromatography [18].

2.4. Statistical analysis

Study data were analyzed using Statistics for Windows 5.1 (Statsoft Inc., Tulsa, OK, USA) and SAS for Windows. Differences in baseline parameters were tested using two sample *t*-tests. Fisher's Exact test was used to test the proportions of participants in each treatment group who were above or below the overall median percentage change in hot flush count from the week prior to randomization to week 4, 8 and 12 (study end). The median was used because the data were not normally distributed, and the median therefore, provided a more accurate representation of the data. Efficacy data recorded after randomization were used for analysis. The evaluable sample included 11 datasets from the placebo group and 15 datasets from the isoflavones group. Analysis was according to Intent-to-Treat (ITT).

3. Results

Forty-two women were screened and 30 were randomized during the 6 months screening period (16 to active treatment and 14 to placebo). Six participants were ineligible due to < 5 hot flushes per day, four did not return to the clinic and two recorded inadequate data on the diaries. The age, weight, height and body mass index (BMI) of the randomized group are shown in Table 1 and show no significant differences between the two groups at randomization. Three women withdrew from the isoflavone group and three from the placebo group. Principal reason for withdrawal was lack of efficacy. Tolerability was generally good and the active group showed no more side effects than the placebo group.

3.1. Hot flushes

There was no difference in the hot flush count between the two groups at baseline. The primary endpoint assessed was the reduction in hot flushes from baseline to 12 weeks treatment. In the single blind run-in, for all patients, there was a median decrease in the number of hot flushes from 6 to 5 (−16.7%). When the results from the 4 weeks run-in were grouped according to the prospective treatment or placebo arm in the double blind phase, there was no statistically significant difference in decrease of hot flushes for the group later randomized to isoflavones than for the group later randomized to placebo group.

The median values for the percentage change throughout the 12-week study for the placebo and isoflavones treated groups of the double blind phase are shown in Fig. 1.

The median percentage change of hot flushes for placebo remained close to baseline over the 12 weeks, while the median change for isoflavones reduced by 44% over the same period. The sharpest decline occurred between weeks 1 and 3 with a decrease of −33%. Hot flushes continued to decrease to a maximum of −56% at week 10, and then leveled at −44% at weeks 11 and 12. Fisher's exact test demonstrated significance at

Table 1
Baseline characteristics of study participants

	Placebo ($n = 14$)	80 mg Promensil ($n = 16$)	P^a
Age at trial start	52.5 ± 5.2 years	54.2 ± 7.4 years	0.2
Weight	67.8 ± 8.8 kg	70.6 ± 15.2 kg	0.56
Height	165.2 ± 6.7 cm	163.4 ± 5.8 cm	0.44
BMI	24.8 ± 3	26.4 ± 5.4	0.35

Values are given as mean ± SD.
[a] *P* values calculated from Student's *t*-test.

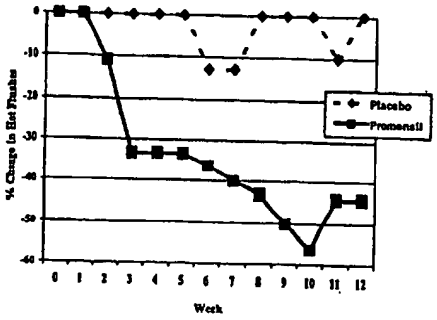

Fig. 1. Median percentage change in hot flushes during the 12 weeks double blind phase*. *Median values for all data at W4 = 0, W8 = −22.6 and W12 = −26.7%. The difference in the proportion of patients above and below the median was tested at weeks 4, 8 and 12. It was significant using the Fisher's Exact Test between placebo and active at weeks 8 and 12 ($P = 0.0154$).

week 8 for a median of −22.5% change in hot flushes from baseline ($P = 0.0154$) and week 12 for the median of −26.7% change ($P = 0.0154$). Only two of 11 (18%) placebo participants responded below the median, while 11 of 15 (73%) isoflavone users responded at week 12. The mean values for the hot flush count are shown in Table 2.

3.2. Greene score

Overall menopausal symptoms, as measured by the Greene score, were observed to be slightly reduced in the active group while the overall score for the placebo group slightly increased (Table 2).

The difference between groups was not large enough to reach a level of significance.

3.3. Urinary isoflavone excretion

Total isoflavone excretion did not change significantly among women in the placebo group, however excretion of total isoflavone increased significantly for women in the active treatment group from baseline to study end ($P = 0.0005$); and from randomization to study end ($P = 0.027$) (Table 3).

Data was graphed for the individual and total isoflavone levels against the mean hot flush count for the week at which urine was collected. There were three urine collections per participant. Total urinary isoflavone excretion (mg/24 h) values against the hot flush count (per 24 h) are shown as Fig. 2. Statistical or correlation analysis was not appropriate because the data were not independent. Graphical representation of each individual isoflavone against the hot flush count revealed similar trends but are nor shown.

4. Discussion

This study demonstrates that following 12 weeks treatment with 80 mg isoflavones (Promensil) the hot flush count reduced by 44% while there was no further change in hot flush frequency of the placebo group. The difference was statistically significant at both weeks 8 and 12. Other menopausal symptom studies have revealed a high

Table 2
Change in Greene score and hot flush count

	Randomization	Week 4	Week 8	Week 12
Greene score				
Placebo ($n = 11$)	13.75 ± 9.5	15.08 ± 12.87	13.45 ± 11.6	14.55 ± 11.8
Promensil ($n = 15$)	12.5 ± 11.2	11.73 ± 8.06	11.27 ± 8.5	10.9 ± 9.89
Hot flushes				
Placebo ($n = 11$)	5.75 ± 5	5.32 ± 3.3	5.9 ± 4.9	6.04 ± 5.5
Promensil ($n = 15$)	5.43 ± 2.6	4.53 ± 3.4	3.74 ± 2.9	3.35 ± 3

Data are presented as mean ± SD. The Greene score is a 21-symptom measure, which does not include the number of hot flushes. Hot flushes are shown separately and are the weekly average of the number of hot flushes per day.

Table 3
Mean isoflavone excretion (mg/24 h) by treatment group at baseline, week 4, and end of study (week 12)

	Baseline	Randomization	P	Week 12	P
Placebo (±SD)	0.3 ± 0.6	1.1 ± 2	NS	2.7 ± 4.2	NS
Promensil (±SD)	0.3 ± 0.5	1.8 ± 3.3	NS	5.2 ± 3.8	0.0005[a]

SD, standard deviation; NS, not significant from baseline.
[a] Significant difference from baseline (Student's *t*-test).

placebo effect [20]. Use of a placebo tablet during the run-in phase to identify participants that might be partial to its influence has been suggested as a way of normalizing the study group by accounting for potential spurious inaccuracies at the outset. The handling of the study in this way may allow one to better concentrate on the outcomes as treatment specific effects. This strategy has been employed in studies of hypertension [21], diabetes [22], asthma [23], premenstrual syndrome [24], and psychological studies [25]. The lack of placebo effect during the double blind treatment phase may be explained by the 16% reduction of hot flushes occurring during this type of run-in phase which is equivalent to the level of the placebo effect on hot flush reduction in other studies using non-prescription therapies [8,10].

While only 18% of the placebo participants reported less severe hot flushes after 12 weeks, 73% of the Promensil participants reported an improvement at weeks 8 and 12. These results were confirmed by the study of Jeri and Romana [12] where the treatment group reported a statistically significant reduction of 43.9% in the frequency of hot flushes per day compared to 5.5% in the control group. Other factors, which may have contributed to the results of the current study when compared to other studies that were done with isoflavones, are that in this study 80 mg of isoflavones per day was used, while in other studies 40 mg was given. Also the entry criteria of more than five hot flushes per day rather than three might have been of influence. The only study using a soy preparation and showing a statistically significant result did include women with at least seven hot flushes per day [26]. Drawbacks in prior negative, placebo-controlled, trials with Promensil [8,9] might have been that in these studies mildly symptomatic or perimenopausal individuals were included and that the dietary intake of isoflavones was not controlled. We believe the latter to be an important point as in order to attribute any improvement of the measured symptom; subjects must not be allowed to obtain isoflavones externally during the placebo phase. Part of the apparent placebo effect in many previous trials of hot flush incidence has been attributed to inadvertent or deliberate intake of isoflavone-rich foods during the trial period, even when a higher dose of 160 mg per day is used [9]. In this trial, the absence of a placebo effect at weeks 4, 8, and 12 is consistent with this proposition as the intake of isoflavone-containing foods was strictly controlled in the placebo group and in any case the Dutch population have low background levels of isoflavones in their diet. Studies of prescription products generally are not affected by these considerations, as potential participants in placebo groups would not have access to the test compounds from the natural environment. A further potential confounding factor was raised in a report of a recent estrogen trial, which reported that perimenopausal women have a higher placebo effect than postmenopausal women [27]. Such issues must be taken into consideration when designing any study assessing the effectiveness of dietary supplementation. Results of previous clinical studies assessing the effects of isoflavones on menopausal symptoms have been varied but there has been epidemiological, anecdotal and clinical evidence to support the use of isoflavones for hot flushes. Although, the effects in this regard have not been as profound as those observed with HRT, it is important that there has been no evidence of adverse estrogenic effects on the endometrium with red clover isoflavones

[18,28], alleviating concerns of undesirable side effects associated with these prescription therapies. A recent single case report of a woman diagnosed with endometrial cancer and a history of taking supplements (some possibly containing phytoestrogens) suggested that additional research is needed concerning endometrial safety [19].

Since isoflavone bioavailability varies between individuals and access to food-sourced isoflavones is difficult to control, a more accurate way of demonstrating the physiological effectiveness of isoflavone-based therapies may be through analyzing excretion (as a reflection of intake) versus specific symptom or laboratory parameters. The graph shows that participants, who absorbed and metabolized greater amounts of isoflavones, had lower hot flush counts. This has also been reported in another red clover study [8] and in a more recent Japanese community-based prospective study [29]. Different reasons may account for the variability in absorption [30]. Some studies have shown that people with higher dietary intakes of carbohydrates and less fat produced higher levels of metabolites [31]. Gut flora are also an important part of isoflavone metabolism as bioavailability depends on an individual's gut microflora [32]. It would be interesting to assess in larger future studies the relationship between isoflavone correlations and menopausal symptoms. This study indicates the view that the use of a red clover isoflavone dietary supplement is effective in alleviating the acute hot flush symptoms of menopause. While isoflavones can be obtained from the diet, dietary modification is a difficult option because of the need to ingest large quantities of legume food plants and the variability in intake. Although, a range of isoflavone dietary supplements are now available, those produced from red clover offer the benefit of containing all four important isoflavones and Promensil provides these in a form standardized with respect to concentration and ratio.

Acknowledgements

This study was funded by Novogen Ltd, North Ryde, Australia. The authors are indebted to Leanne West for her excellent assistance in the preparation of the manuscript and to I Smallegange, Research-Nurse. Department of Obstetrics and Gynaecology, Gelre Hospital, Apeldoorn, The Netherlands for her assistance in conducting the study.

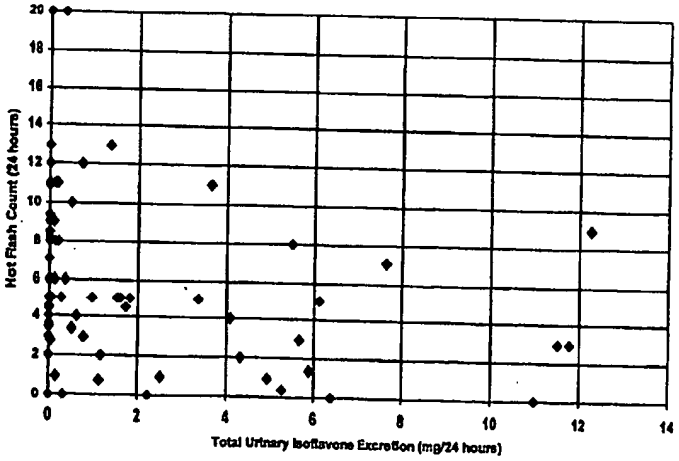

Fig. 2. Graph of hot flush count over 24 h and total urinary isoflavone excretion (mg/24 h). Three urine samples per patient at three points throughout the study.

D16

ORIGINAL CONTRIBUTION

Phytoestrogen Supplements for the Treatment of Hot Flashes: The Isoflavone Clover Extract (ICE) Study

A Randomized Controlled Trial

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Context Clinical trials demonstrating increased risk of cardiovascular disease and breast cancer among women randomized to hormone replacement therapy have increased interest in other therapies for menopausal symptoms. Dietary supplements containing isoflavones are widely used as alternatives to hormonal therapies for hot flashes, but there is a paucity of data supporting their efficacy.

Objective To compare the efficacy and safety of 2 dietary supplements derived from red clover with placebo in symptomatic menopausal women.

Design, Setting, and Participants Randomized, double-blind, placebo-controlled trial of menopausal women, aged 45 to 60 years, who were experiencing at least 35 hot flashes per week. The study was conducted between November 1999 and March 2001 at 3 US medical centers and included women who were recently postmenopausal (mean [SD], 3.3 [4.5] years since menopause) experiencing 8.1 hot flashes per day. Women were exluded if they were vegetarians, consumed soy products more than once per week, or took medications affecting isoflavone absorption.

Intervention After a 2-week placebo run-in, 252 participants were randomly assigned to Promensil (82 mg of total isoflavones per day), Rimostil (57 mg of total isoflavones per day), or an identical placebo, and followed-up for 12 weeks.

Main Outcome Measure The primary outcome measure was the change in frequency of hot flashes measured by participant daily diaries. Secondary outcome measures included changes in quality of life and adverse events.

Results Of 252 participants, 246 (98%) completed the 12-week protocol. The reductions in mean daily hot flash count at 12 weeks were similar for the Promensil (5.1), Rimostil (5.4), and placebo (5.0) groups. In comparison with the placebo group, participants in the Promensil group (41%; 95% confidence interval [CI], 29%-51%; $P=.03$), but not in the Rimostil group (34%; 95% CI, 22%-46%; $P=.74$) reduced hot flashes more rapidly. Quality-of-life improvements and adverse events were comparable in the 3 groups.

Conclusion Although the study provides some evidence for a biological effect of Promensil, neither supplement had a clinically important effect on hot flashes or other symptoms of menopause.

JAMA. 2003;290:207-214 www.jama.com

HOT FLASHES ARE THE PRIMARY reason that women seek medical attention for menopausal symptoms. The recent results from the Women's Health Initiative[1] and long-term follow-up from the Heart and Estrogen/progestin Replacement Study[2,3] demonstrating an increased risk of cardiovascular disease and breast cancer among women randomized to hormone therapy are likely to reduce the use of hormones for relief of menopausal symptoms. Dietary supplements containing isoflavones derived from soy or red clover are heavily marketed as alternative treatments for menopausal symptoms. In Asia, only 10% to 20% of women experience hot flashes compared with 70% to 80% of women in Western countries.[4-7] A popular hypothesis to explain this difference is that isoflavones found in soy, a staple in the traditional Asian diet, influence the body's response to the changing hormonal levels of menopause.[8] Isoflavones are polyphenol compounds structurally related to estrogens that have been shown to bind to estrogen receptors[9] and appear to act as partial agonists in some tissues and antagonists in others. They have a higher binding af-

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finity for estrogen-receptor β than for estrogen-receptor α.[10-12]

Dietary supplements containing isoflavones from soy or red clover are widely marketed for menopausal symptoms and are increasingly being used by women in the United States as an alternative to estrogen.[13-16] Most published studies of isoflavones for relief of menopausal symptoms have investigated the effectiveness of soy products.[17-28] Dietary supplements derived from red clover contain additional isoflavones (biochanin A, formononetin) not found in soy, which may have additional biological activity. On the other hand, red clover lacks components of soy that may contribute to soy's biological effects. There are few published data[29-33] and no published large clinical trials on the effects of these compounds on menopausal symptoms and quality of life. Furthermore, many clinicians and the public have expressed concern about the safety of dietary supplements.

We initiated the Isoflavone Clover Extract study to investigate whether 2 dietary supplements derived from red clover were safe and more effective than placebo at reducing hot flashes and improving menopausal quality of life in symptomatic postmenopausal women.

METHODS

Participants

Women were recruited at 3 academic clinical research sites located in Oakland, Calif; Minneapolis, Minn; and Iowa City, Iowa. The study was administered through a coordinating center at the University of California, San Francisco. The institutional review boards at each clinical site and at the coordinating center approved the study protocol. All participants gave written informed consent.

Women were recruited from the general population through newspaper and radio advertising, flyers posted in clinics and at health fairs, and directed mailings. Participants were enrolled between November 1999 and November 2000. All participants were aged 45 to 60 years, experiencing at least 35 hot flashes per week, and had a follicle-stimulating hormone (FSH) level of 30 mIU/mL. Eligible women had either documented bilateral oophorectomy or at least 2 consecutive months of amenorrhea prior to enrollment with at least 6 months of amenorrhea in the year prior to entry. Women were excluded from the study if they were vegetarian, consumed soy products more than once per week, took medications affecting isoflavone absorption (antibiotics, antacids) or hormonal preparations during the 3 months prior to enrollment, had significant gastrointestinal disease, drank more than 2 alcoholic beverages per day, were allergic to red clover, were regular users of dietary supplements containing isoflavones, or consumed less than 80% of the expected study tablets during the 2-week placebo run-in period.

Study Supplements and Randomization

The 2 study supplements, Promensil and Rimostil, and identical placebo were prepared by the manufacturer (Novogen Ltd, Sydney, Australia) and sent to a central research pharmacy for packaging and labeling. The central pharmacy was not involved in the study design or participant monitoring. Promensil contains a higher proportion of biochanin A and genistein. Rimostil contains a higher proportion of formononetin and daidzein. An independent laboratory (Sigma Pharmaceuticals, South Croydon, Australia) verified the contents of the study tablets. Placebo tablets contained less than 0.04 mg of total isoflavones per tablet; Promensil tablets contained an average of 41.0 mg of total isoflavones (range, 37.0-43.0 mg); and Rimostil tablets contained an average of 28.6 mg of total isoflavones (range, 25.6-31.4 mg). Participants were instructed to take 2 tablets once daily.

The randomization schedule was prepared by the central pharmacy using computer-generated randomization in blocks of 6, stratified by clinical site. The allocation schedule was maintained at the pharmacy. Each site received numbered containers and distributed them sequentially at randomization. The clinical center principal investigators, their staff, the participants, and the coordinating center principal investigator and staff were all blinded to treatment allocation until the last participant completed her close-out visit and the data clean-up was finished.

Measurements

Staff from each of the clinical sites attended a training session organized by the coordinating center to ensure standard administration of the study protocol and to certify staff on measurement techniques. Participant eligibility, according to the selection criteria previously described, was assessed at an initial screening telephone call and 2 clinic visits. At the first clinic visit, weight, height, pulse, and blood pressure were measured according to a standard protocol. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Demographics, reproductive history, smoking, and alcohol consumption were assessed by participant self-report. A supply of placebo tablets was distributed for a 2-week run-in phase. The participants were informed that the run-in tablets were placebos and were the same as those that were to be used in the main study. At the end of the run-in phase, baseline questionnaires and physical examination were completed. A 24-hour urine sample was collected and willing participants, who were at least 80% compliant with the run-in regimen, were randomized to receive a dietary supplement or placebo.

Participants were contacted by telephone at 1, 4, and 8 weeks to encourage compliance, assess adverse effects, verify concurrent medications, and obtain follow-up information on hot flashes and other symptoms. At 12 weeks, the trial participants returned to the clinic sites for a full evaluation including repeat of the quality-of-life measures, physical examination, blood draw, and 24-hour urine collection. Compliance was assessed by pill count.

Women participating in the study were given hot flash diary cards to record the number of hot flashes and night sweats they experienced on a daily basis.[34] Hot flash counts were averaged for each week. If more than 3 days during a week had missing data, the average for that week of the study was treated as missing.

Changes in quality of life were assessed using the Greene Climacteric Scale, a validated instrument for women experiencing symptoms attributed to menopause.[35] This instrument has 6 subscales specifically designed to assess menopausal symptoms. The Greene questionnaire was completed at randomization and at 1, 4, 8, and 12 weeks after randomization.

Fasting serum, 24-hour urine collections, and second morning void urine specimens were collected prior to randomization and at study closeout. All specimens were aliquoted at the clinical sites, frozen at –80°C, and sent to a central laboratory for storage (Esoterix Inc, Calabas Hills, Calif). Paired 24-hour urine specimens were analyzed for isoflavone excretion (genistein, daidzein, biochanin A, formononetin, o-desmethyl-angolensin, and equol) by laboratory personnel blinded to treatment allocation (Australian Government Analytical Laboratories, Canberra). Total isoflavone excretion was calculated as the sum of the individual isoflavone excretion amounts.

Statistical Considerations

We hypothesized that the isoflavone supplements would be more effective than placebo in reducing hot flashes. The study was designed to have 90% power to detect at least a 15% greater reduction in hot flash frequency in the active treatment arms compared with the placebo arm. We assumed that women taking placebo would have a 25% decrease in the number of weekly hot flashes.

We analyzed differences in rate of change of weekly hot flash counts over the 12-week treatment period using a random coefficients regression model with a quadratic effect for each treatment through time. Each participant had her own random intercept and slope. Separate analyses were done comparing each phytoestrogen supplement with placebo. No analysis combining the 2 active treatment arms was planned or performed. The primary analysis was an intention-to-treat analysis that included all patients who were randomized. No adjustment for baseline covariates was planned for the primary analysis. Models were also analyzed including covariates known to be associated with hot flashes. A secondary per protocol analysis was performed, which included only participants who had hot flash count data available for the 12th week after randomization, who had at least 80% compliance with study tablets by pill count, and whose total isoflavone excretion was less than 1 mg/24 hours at baseline and remained less than 1 mg/24 hours at closeout (placebo group) or was more than 1 mg/24 hours (phytoestrogen groups). The remaining prespecified subgroups were time since menopause (5 years vs >5 years), BMI (median vs >median), and FSH level (median vs >median). They were analyzed to identify women who might particularly benefit from either phytoestrogen supplements. Because isoflavone excretion was not normally distributed, Spearman correlation was used to assess the association of change in hot flash number with change in urinary isoflavone excretion.

Scores for the subscales of the Greene Climacteric Scale were calculated using the standard method described by Greene.[35] Data are reported using the last observation carried forward. Alternative strategies for imputation of missing values did not affect the results nor did per protocol (secondary) analyses.

Baseline characteristics were summarized by treatment group. For continuous variables, means were compared using analysis of variance for normally distributed variables and the Kruskal-Wallis test for variables with skewed distributions. Categorical variables were compared using the χ^2 test. For all primary and secondary outcomes, outliers were included in the principal analysis. Secondary analyses excluding participants with values higher than 3 SDs from the mean did not alter the results and have not been presented in this article. Safety data was tabulated according to initial randomization assignment. We reported all adverse events occurring in at least 3% of the women and those that differed across arms ($P=.05$). The Fisher exact test was used to examine the difference in rates of occurrence between the 3 groups. A 2-tailed P value of less than .05 was considered statistically significant for all analyses.

RESULTS

Participants

Among 1191 women screened by telephone (FIGURE 1), 870 were ineligible. The principal reasons for ineligibility included too few hot flashes (n=223), not interested in participation (n=205), medical conditions and medications (n=192), dietary exclusions (n=104), and not being menopausal (n=94). Of the 321 women who were invited to the clinic for blood tests and a 2-week placebo run-in, 69 were ineligible. The principal reasons for ineligibility included too few hot flashes (n=28), FSH level of less than 30 mIU/mL (n=18), and not interested in participation (n=11). Only 3 women were ineligible for randomization due to inadequate adherence during the run-in. Participants were randomized to Promensil (n=84), Rimostil (n=83), or placebo (n=85). All participants received treatment as allocated. Two participants in each arm did not complete the 12-week active phase of the study.

At baseline, the participants did not differ across groups by age, demographic characteristics, reproductive factors, or FSH level (TABLE 1; all $P>.05$). On average, the women were recently postmenopausal, experiencing about 8 hot flashes per day, and were white.

Compliance

Ninety-eight percent (246/252) of the women completed the full 12 weeks of the study. The participants took 97%

of the expected number of tablets by count of the returned tablets, and 98% of the participants took at least 80% of the tablets. Compliance did not differ across groups (P=.21). Only 1 woman dropped out because of an adverse event (nausea in a participant randomized to Rimostil).

Hot Flashes

The reduction in mean hot flash count at 12 weeks was 41% (95% confidence interval [CI], 29%-51%) for the Promensil group, 34% (95% CI, 22%-46%) for the Rimostil group, and 36% (95% CI, 26%-45%) for the placebo group (TABLE 2). The change in hot flash counts from randomization to closeout was significant for all 3 groups (P<.001). However, the hot flash reductions in the phytoestrogen groups were not statistically different from placebo at 12 weeks (P>.20). The reduction in hot flashes was faster for Promensil compared with placebo (FIGURE 2; P=.03). The comparable analysis for Rimostil vs placebo found that the rate of reduction in hot flashes was similar for the 2 groups (P=.74). Adjusting for baseline covariates including study site, season, FSH level, age at randomization, age at menopause, and time since menopause did not change the results. On average, women in all 3 groups were still experiencing more than 5 hot flashes per day at the end of the 12-week study period.

Per protocol results (n=197) were similar to the intention-to-treat analyses. There was some evidence that the benefit of the phytoestrogen supplements in reducing hot flash frequency was most pronounced for women above the median BMI. For Promensil, the reduction in hot flashes over 12 weeks was 49% (95% CI, 35%-63%) for women above the median BMI (25.1) and 30% (95% CI, 16%-44%) for thinner women (BMI <25) (P for interaction=.09). For Rimostil, the reduction in hot flashes over 12 weeks was 45% (95% CI, 32%-59%) for overweight women and 22% (95% CI, 7%-37%) for thinner women (P for interaction=.02). For women in the placebo group, the reduction in hot flashes over 12 weeks was 32% (95% CI, 21%-42%) for overweight women and 40% (95% CI, 26%-55%) for thinner women. There were no significant interactions for the subgroups defined by FSH level or years since menopause.

Among the 241 women with paired 24-hour urine results available, there was no statistically significant correlation of change in hot flash number with change in total isoflavone excretion (ρ=.01; P=.84) or with change in the excretion of genistein, daidzein, bio-

Figure 1. Flow Diagram of Isoflavone Clover Extract Study

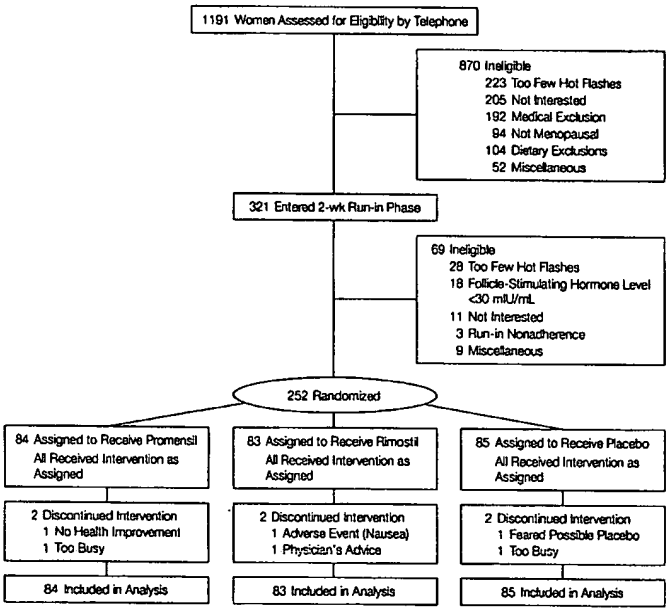


Table 1. Clinical and Demographic Characteristics at Baseline

	Promensil (n = 84)	Rimostil (n = 83)	Placebo (n = 85)
	Mean (SD)		
Age, y			
Current	52.3 (2.8)	52.3 (3.0)	52.3 (3.4)
At menopause	49.1 (4.5)	48.6 (5.2)	49.5 (4.9)
Time since menopause, y	3.3 (4.3)	3.9 (5.1)	2.8 (4.1)
Body mass index	26.3 (5.1)	25.6 (4.2)	26.5 (5.4)
Follicle-stimulating hormone level, mIU/mL	80 (30)	85 (33)	81 (35)
Hot flashes per day	8.5 (4.8)	8.1 (3.0)	7.8 (2.4)
	No. (%)		
Surgical menopause	6 (7)	4 (5)	6 (7)
>High school education	34 (40)	31 (37)	40 (47)
Current smoker	14 (17)	5 (6)	10 (12)
Race or ethnicity			
White	71 (85)	73 (88)	69 (81)
Black	10 (12)	8 (10)	7 (8)
Other	3 (4)	2 (2)	9 (11)

Table 2. Hot Flashes per Day at Baseline and During Study Treatment

	Promensil (n = 84)		Rimostil (n = 83)		Placebo (n = 85)	
Week	Baseline, Mean (95% CI)	% Reduction From Baseline (95% CI)	Baseline, Mean (95% CI)	% Reduction From Baseline (95% CI)	Baseline, Mean (95% CI)	% Reduction From Baseline (95% CI)
0	8.5 (7.4-9.5)	0	8.1 (7.4-8.7)	0	7.8 (7.3-8.3)	0
4	6.2 (5.0-7.4)	27 (13-41)	5.9 (5.1-6.7)	27 (17-37)	6.2 (5.5-6.9)	21 (12-29)
8	5.2 (54.1-6.3)	38 (26-52)	5.9 (5.0-6.8)	27 (16-38)	5.4 (4.7-6.2)	31 (21-40)
12	5.1 (4.2-6.0)	41 (29-51)	5.4 (4.4-6.3)	34 (22-46)	5.0 (4.3-5.8)	36 (26-45)

Abbreviation: CI, confidence interval.

chanin A, formononetin, o-desmethylangolensin, or equol.

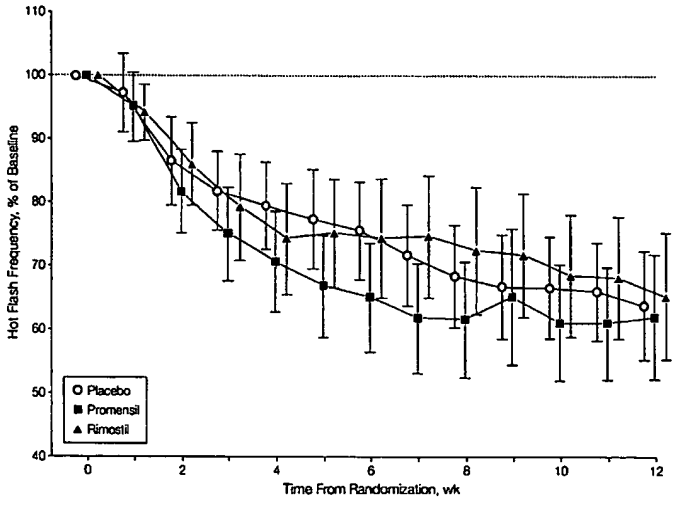
Greene Symptom Scales

Compared with age- and sex-matched population normative data,[36] women entering this study reported high levels of distress on the vasomotor scale (mean [SD], 3.6 [1.2]), but lower distress on the psychological (mean [SD], 6.0 [4.4]) and somatic (mean [SD], 2.8 [2.4]) scales. Over the 12-week treatment period, there were significant improvements from baseline in all 3 groups, but there were no statistically significant differences between groups on any of the Greene scales (TABLE 3).

Adverse Events

Adverse events occurring in at least 3% of the participants are shown in TABLE 4. There was no statistically significant association of either of the dietary supplements with adverse events; the only adverse event approaching statistical significance was headache ($P=.10$ for Rimostil vs placebo; $P=.12$ for Promensil vs placebo), which was more common among women randomized to placebo. There was no association with vaginal spotting (3.6% for Promensil group, 1.2% for Rimostil group, and 2.4% for placebo group) and there were no reports of breast tenderness, venous thrombosis, pulmonary embolism, myocardial infarction, stroke, fracture, or gallbladder disease. There were no treatment-related changes in weight, blood pressure, or heart rate ($P=.28$).

Figure 2. Hot Flash Frequency as Percentage of Baseline



COMMENT

The Isoflavone Clover Extract study was a large, multicenter, randomized, placebo-controlled trial of red clover extracts in postmenopausal women reporting hot flashes. At the end of 12 weeks, the reduction in hot flashes was similar for the 3 groups. Promensil, but not Rimostil, reduced the frequency of hot flashes more rapidly than placebo. The reduction was modest (41% over 12 weeks), but similar in size to that found in other studies of phytoestrogen supplements.[17-29,37]

There were no significant differences for either supplement compared with placebo in 6 domains of menopause symptoms assessed at regular intervals by the Greene Climacteric Scale. However, all 3 groups reported improvements in their symptom scores. The magnitude of these changes were between 40% and 94% of the differences reported between perimenopausal women and premenopausal women in a population-based study validating these scales.[36] This suggests that women in all 3 arms experienced improvements in quality of life that were clinically important. The magnitude of the improvements in hot flashes and other menopausal symptoms in the placebo group highlight the importance of placebo-controlled clinical trials in evaluating the efficacy of potential therapies for menopausal symptoms.

The red clover extracts were well tolerated by the participants. We did not find any trend toward an association of these dietary supplements with adverse outcomes. However, the 12-week intervention period was too short to assess the risk for endometrial hyperplasia, breast cancer, venous throm-

Table 3. Change in the Greene Climacteric Subscales From Randomization to the End of Study

	Promensil (n = 83)		Rimostil (n = 82)		
	Mean (95% CI)	Promensil vs Placebo *P* Value*	Mean (95% CI)	Rimostil vs Placebo *P* Value*	Placebo (n = 84), Mean (95% CI)
Psychological	−1.8 (−2.6 to 0.9)	.23	−1.2 (−2.0 to 0.3)	.77	−1.0 (−1.9 to 0.1)
Anxiety	−1.1 (−1.6 to 0.6)	.33	−0.8 (−1.3 to 0.3)	.80	−0.7 (−1.3 to 0.2)
Depression	−0.7 (−1.1 to 0.2)	.23	−0.4 (−0.8 to −0.2)	.79	−0.3 (−0.7 to −0.2)
Somatic	−0.4 (−0.8 to −0.03)	.60	−0.6 (−1.1 to 0.2)	.82	−0.6 (−1.0 to 0.1)
Vasomotor	−1.1 (−1.5 to 0.8)	.93	−0.9 (−1.3 to 0.6)	.36	−1.2 (−1.5 to 0.8)
Sexual desire	−0.1 (−0.2 to −0.1)	.23	−0.2 (−0.3 to −0.03)	.66	−0.2 (−0.4 to 0.02)

Abbreviation: CI, confidence interval.
*t Test used (last observation carried forward analysis).

Table 4. Adverse Events

	No. (%) of Participants			
Adverse Event	Promensil (n = 84)	Rimostil (n = 83)	Placebo (n = 85)	*P* Value
Any	31 (37)	28 (34)	33 (39)	.80
Cold or upper respiratory tract infection	9 (11)	10 (12)	14 (16)	.57
Headache	5 (6)	4 (5)	11 (13)	.13
Myalgia	10 (12)	8 (10)	7 (8)	.73
Nausea	4 (5)	8 (10)	4 (5)	.34
Arthralgia	5 (6)	7 (8)	6 (7)	.79
Diarrhea	2 (2)	3 (4)	3 (4)	.91

boembolic disease, cardiovascular disease, or other potentially serious adverse events, which have been associated with estrogenic therapies. Potential long-term benefits also could not be evaluated. Furthermore, with only 252 participants followed-up for 3 months, we had limited power to detect rare adverse events.

Most prior studies of isoflavones have used soy products.[17-28] The results have been mixed: some studies found a modest benefit (10%-15%) compared with placebo[17-19,21,24,25,32,33] and others did not.[20,22,23,26-29] A small, uncontrolled study of a red clover extract reported a 56% decrease in hot flashes.[31] Two small randomized clinical trials of the same extract found more modest reductions in hot flash frequency (22%-34%) that were similar to those observed in the placebo group,[29,30] but 2 more recent randomized trials have reported statistically significant reductions of 44%[33] and 49%.[32] Several studies have suggested that once daily consumption of isoflavones is not sufficient for optimal symptom relief given that the half-life of isoflavones is between 6 and 10 hours.[38-40] This may explain some of the heterogeneity in the results: a twice-a-day dose schedule might be efficacious. There has also been a wide range in the amount of total isoflavones used in these studies. Although the amount of total isoflavones used in the Isoflavone Clover Extract study were relatively high, it is possible that higher doses are needed to have a clinically important effect. The reduction in hot flashes reported in the positive studies (40%-54%)[14-16,18,21,22,32] has been smaller than the 77% reduction reported in a meta-analysis of clinical trials using hormone replacement therapy.[41] Furthermore, a randomized clinical trial of soy found that adding 0.625 mg of conjugated equine estrogens after 6 weeks of soy therapy resulted in a statistically significant additional reduction in hot flashes.[24] As in the present study, those who have demonstrated a statistically significant reduction in hot flashes with phytoestrogens generally report a tendency toward early benefit that decreases after 8 to 12 weeks of therapy.[19,25]

The mechanism of action of these supplements is unclear but is thought to be primarily through estrogenlike effects of the isoflavones. Isoflavones are structurally similar to estradiol, binding to both estrogen-receptor α and estrogen-receptor β, and appear to have tissue-specific effects like selective estrogen-receptor modifiers.[9-12] They have been shown to affect the catabolism of estrogens[42] and may affect estrogen-receptor expression.[43] Several nonhormonal mechanisms have been demonstrated for isoflavones including tyrosine kinase inhibition, antioxidant activity, and effects on ion transportation.[44]

Given that the overall reduction in hot flashes was not different between the 3 treatment groups and that the effect of isoflavone supplementation on rate of reduction was seen only for Promensil and not Rimostil, it could be argued that the results are due to chance alone and not the biological effects of the isoflavones. Alternatively, the results may indicate that the biochanin A and genistein found in higher concentrations in Promensil are more effective for hot flash reduction than the formononetin and daidzein found in Rimostil. Genistein is also present in higher concentrations than daidzein in soy, which is the basis of the traditional Asian diet.[45,46]

We examined several subgroups to explore whether certain populations might receive greater benefit from isoflavones derived from red clover. Heavier women appeared to receive more benefit from the isoflavone supplements while the changes in the

placebo group were similar to a placebo effect. This was contrary to our expectation and needs to be reproduced in other studies. Postmenopausal women with higher BMIs tend to have higher circulating estrogens due to conversion of androgens to estrogens by aromatase in adipocytes.[47] We hypothesized that the isoflavones might have greater effects in an estrogen-poor environment, although the relationship between estrogen level and menopausal symptoms has been inconsistent.[47,48] Several studies have reported a higher incidence of hot flashes in women with higher BMIs.[49,50] This may be due to the insulating effects of body fat leading to a more rapid rise in core body temperature, which then triggers hot flashes.[51] It is unclear why phytoestrogens would have greater efficacy in overweight women.

This study has several limitations. Most of the study participants were white and highly educated, which limits the generalizability of the results to other socioeconomic or racial groups. In addition, the women were all postmenopausal. Thus, the results may not apply to perimenopausal women, which is usually the period when women experience the most frequent and severe hot flashes. Furthermore, we required women to document at least 35 hot flashes per week to be eligible for this study; less symptomatic women may or may not benefit.

This study is the largest randomized clinical trial of red clover extracts in postmenopausal women. Compliance with therapy was exceptionally high (98%) and the drop-out rate was low (2%). We attempted to recruit from a broad cross-section of the population through media advertising and mailings to age-eligible women, rather than recruiting primarily from menopause clinics or referral centers.

In conclusion, the overall reduction in hot flashes after 12 weeks of treatment was modest and similar between women in all 3 groups. Promensil reduced hot flashes more rapidly than placebo. Although the study provides some evidence for a biological effect of Promensil, neither supplement had a clinically significant effect on hot flashes or other menopausal symptoms when compared with placebo.

Author Contributions: *Study concept and design:* Tice, Ettinger, Wallace, Cummings.
Acquisition of data: Tice, Ettinger, Ensrud, Wallace.
Analysis and interpretation of data: Tice, Ettinger, Ensrud, Wallace, Blackwell, Cummings.
Drafting of the manuscript: Tice, Wallace, Blackwell.
Critical revision of the manuscript for important intellectual content: Ettinger, Ensrud, Wallace.
Statistical expertise: Blackwell.
Obtained funding: Tice, Ensrud, Wallace.
Administrative, technical, or material support: Wallace.
Study supervision: Ettinger, Wallace, Cummings.
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Funding/Support: The Isoflavone Clover Extract study was funded by a grant from Novogen Inc.
Acknowledgment: We acknowledge Charles McCulloch, PhD, for statistical guidance, and the staff of the individual sites for their hard work and devotion to quality.

REFERENCES

1. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. *JAMA.* 2002;288:321-333.
2. Grady D, Herrington D, Bittner V, et al, for the HERS Research Group. Cardiovascular disease outcomes during 6.8 years of hormone therapy: Heart and Estrogen/progestin Replacement Study follow-up (HERS II). *JAMA.* 2002;288:49-57.
3. Hulley S, Furberg C, Barrett-Connor E, et al, for the HERS Research Group. Noncardiovascular disease outcomes during 6.8 years of hormone therapy: Heart and Estrogen/progestin Replacement Study follow-up (HERS II). *JAMA.* 2002;288:58-66.
4. Lock M. Contested meanings of the menopause. *Lancet.* 1991;337:1270-1272.
5. Lock M. Menopause in cultural context. *Exp Gerontol.* 1994;29:307-317.
6. Boulet MJ, Oddens BJ, Lehert P, Vemer HM, Visser A. Climacteric and menopause in seven Southeast Asian countries. *Maturitas.* 1994;19:157-176.
7. Tang GW. The climacteric of Chinese factory workers. *Maturitas.* 1994;19:177-182.
8. Adlercreutz H. Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. *Scand J Clin Lab Invest Suppl.* 1990; 201:3-23.
9. Martin PM, Horwitz KB, Ryan DS, McGuire WL. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinology.* 1978;103: 1860-1867.
10. Kuiper GG, Carlsson B, Grandien K, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology.* 1997;138:863-870.
11. Collins BM, McLachlan JA, Arnold SF. The estrogenic and antiestrogenic activities of phytochemicals with the human estrogen receptor expressed in yeast. *Steroids.* 1997;62:365-372.
12. Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology.* 1999;140:5566-5578.
13. The mainstreaming of alternative medicine. *Consumer Reports.* 2000;65:17.
14. Harris PF, Remington PL, Trentham-Dietz A, Allen CI, Newcomb PA. Prevalence and treatment of menopausal symptoms among breast cancer survivors. *J Pain Symptom Manage.* 2002;23:501-509.
15. Kam IW, Dennehy CE, Tsourounis C. Dietary supplement use among menopausal women attending a San Francisco health conference. *Menopause.* 2002;9:72-78.
16. White JP, Schilling JS. Postmenopausal hormone replacement: historical perspectives and current concerns. *Clin Excell Nurse Pract.* 2000;4:277-285.
17. Murkies AL, Lombard C, Strauss BJ, Wilcox G, Burger HG, Morton MS. Dietary flour supplementation decreases post-menopausal hot flushes: effect of soy and wheat. *Maturitas.* 1995;21:189-195.
18. Brzezinski A, Adlercreutz H, Shaoul R, et al. Short-term effects of phytoestrogen-rich diet on postmenopausal women. *Menopause.* 1997;4:89-94.
19. Albertazzi P, Pansini F, Bonaccorsi G, Zanotti L, Forini E, De Aloysio D. The effect of dietary soy supplementation on hot flushes. *Obstet Gynecol.* 1998;91: 6-11.
20. Dalais FS, Rice GE, Wahlqvist ML, et al. Effects of dietary phytoestrogens in postmenopausal women. *Climacteric.* 1998;1:124-129.
21. Washburn S, Burke GL, Morgan T, Anthony M. Effect of soy protein supplementation on serum lipoproteins, blood pressure, and menopausal symptoms in perimenopausal women. *Menopause.* 1999;6:7-13.
22. Kotsopoulos D, Dalais FS, Liang YL, McGrath BP, Teede HJ. The effects of soy protein containing phytoestrogens on menopausal symptoms in postmenopausal women. *Climacteric.* 2000;3:161-167.
23. Quella SK, Loprinzi CL, Barton DL, et al. Evaluation of soy phytoestrogens for the treatment of hot flashes in breast cancer survivors: a North Central Cancer Treatment Group Trial. *J Clin Oncol.* 2000;18: 1068-1074.
24. Scambia G, Mango D, Signorile PG, et al. Clinical effects of a standardized soy extract in postmenopausal women: a pilot study. *Menopause.* 2000;7: 105-111.
25. Upmalis DH, Lobo R, Bradley L, Warren M, Cone FL, Lamia CA. Vasomotor symptom relief by soy isoflavone extract tablets in postmenopausal women: a multicenter, double-blind, randomized, placebo-controlled study. *Menopause.* 2000;7:236-242.
26. St Germain A, Peterson CT, Robinson JG, Alekel DL. Isoflavone-rich or isoflavone-poor soy protein does not reduce menopausal symptoms during 24 weeks of treatment. *Menopause.* 2001;8:17-26.
27. Knight DC, Howes JB, Eden JA, Howes LG. Effects on menopausal symptoms and acceptability of isoflavone-containing soy powder dietary supplementation. *Climacteric.* 2001;4:13-18.
28. Van Patten CL, Olivotto IA, Chambers GK, et al. Effect of soy phytoestrogens on hot flashes in postmenopausal women with breast cancer: a randomized, controlled clinical trial. *J Clin Oncol.* 2002;20: 1449-1455.
29. Baber RJ, Templeman C, Moreton T, Kelly GE, West L. Randomized placebo-controlled trial of an iso-

flavone supplement and menopausal symptoms in women. *Climacteric.* 1999;2:85-92.
30. Knight DC, Howes JB, Eden JA. The effect of Promensil, an isoflavone extract, on menopausal symptoms. *Climacteric.* 1999;2:79-84.
31. Nachtigall L, Fenichel R, La Grega L, et al. The effects of isoflavone derived from red clover on vasomotor symptom, enodmetrial thickness, and reproductive hormone concentrations in menopausal women. Paper presented at: Proceedings of the 81st Annual Meeting of the Endocrine Society; June 12-15, 1999; San Diego, Calif.
32. Jeri A. The use of an isoflavone supplement to relieve hot flushes. *Female Patient.* 2002;27:35-37.
33. van de Weijer P, Barentsen R. Isoflavones from red clover (Promensil) significantly reduce menopausal hot flush symptoms compared with placebo. *Maturitas.* 2002;42:187.
34. Sloan JA, Loprinzi CL, Novotny PJ, Barton DL, Lavasseur BI, Windschitl H. Methodologic lessons learned from hot flash studies. *J Clin Oncol.* 2001;19:4280-4290.
35. Greene J. Constructing a standard climacteric scale. *Maturitas.* 1998;29:25-31.
36. Barentsen R, van de Weijer PH, van Gend S, Foekema H. Climacteric symptoms in a representative Dutch population sample as measured with the Greene Climacteric Scale. *Maturitas.* 2001;38:123-128.
37. Han KK, Soares JM Jr, Haidar MA, de Lima GR, Baracat EC. Benefits of soy isoflavone therapeutic regimen on menopausal symptoms. *Obstet Gynecol.* 2002; 99:389-394.
38. King RA, Bursill DB. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am J Clin Nutr.* 1998;67:867-872.
39. Watanabe S, Yamaguchi M, Sobue T, et al. Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). *J Nutr.* 1998;128:1710-1715.
40. Setchell KD, Brown NM, Desai P, et al. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J Nutr.* 2001;131(4 suppl):1362S-1375S.
41. MacLennan A, Lester S, Moore V. Oral estrogen replacement therapy versus placebo for hot flashes: a systematic review. *Climacteric.* 2001;4:58-74.
42. Xu X, Duncan AM, Merz BE, Kurzer MS. Effects of soy isoflavones on estrogen and phytoestrogen metabolism in premenopausal women. *Cancer Epidemiol Biomarkers Prev.* 1998;7:1101-1108.
43. Fritz WA, Wang J, Eltoum IE, Lamartiniere CA. Dietary genistein down-regulates androgen and estrogen receptor expression in the rat prostate. *Mol Cell Endocrinol.* 2002;186:89-99.
44. Tham DM, Gardner CD, Haskell WL. Clinical review 97: potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J Clin Endocrinol Metab.* 1998; 83:2223-2235.
45. Barnes S, Kirk M, Coward L. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-mass spectrometry. *J Agric Food Chem.* 1994;42:2466-2474.
46. Murphy PA. Phytoestrogen content of processed soybean products. *Food Technol.* 1982;34:60-64.
47. Erlik Y, Meldrum DR, Judd HL. Estrogen levels in postmenopausal women with hot flashes. *Obstet Gynecol.* 1982;59:403-407.
48. Hutton JD, Jacobs HS, Murray MA, James VH. Relation between plasma oestrone and oestradiol and climacteric symptoms. *Lancet.* 1978;1:678-681.
49. Gold EB, Sternfeld B, Kelsey JL, et al. Relation of demographic and lifestyle factors to symptoms in a multi-racial/ethnic population of women 40-55 years of age. *Am J Epidemiol.* 2000;152:463-473.
50. Whiteman MK, Staropoli CA, Langenberg PW, McCarter RJ, Kjerulff KH, Flaws JA. Smoking, body mass, and hot flashes in midlife women. *Obstet Gynecol.* 2003;101:264-272.
51. Freedman RR. Hot flash trends and mechanisms. *Menopause.* 2002;9:151-152.

In the practical use of our intellect, forgetting is as important as remembering.
—William James (1842-1910)

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UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

- - - - - - - - - - - - - - - - X

GRAHAM EDMUND KELLY, :

Junior Party, :

(Application 08/910,837) :

v. : Patent Interference

SHERWOOD L. GORBACH, BARRY R. : No. 104,576

GOLDIN, and HERMAN :

ADLERCREUTZ, :

Senior Party. :

(Patent 5,498,631) :

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Arlington, Virginia

Friday, June 8, 2001

Patent interference hearing (oral argument on preliminary motions for Interference No. 104576) taken at the offices of the U.S. Patent & Trademark Office, 1225 Jefferson Davis Highway, Arlington, Virginia, at 9:00 a.m., Friday, June 8, 2001, and the proceedings being taken down by Stenotype by SUE A. CIMINELLI, and transcribed under her direction.

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JUDGE GARDNER-LANE
JUDGE MEDLEY

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CONTENTS

PROCEEDINGS

JUDGE McKELVEY: This is oral argument on preliminary motions for interference number 104576. You all got my order. We have been through the case rather thoroughly and we have these points that we thought we would bring up, in addition to anything else somebody wants to bring up. And does anybody have an opinion on how we should proceed, because I'm flexible about it.

MR. VOIGHT: I would suggest, Judge McKelvey, that we start with the tutorials.

JUDGE McKELVEY: That's fine. What wants to go first?

MR. O'CONNOR: I would be happy to.

JUDGE McKELVEY: Okay. Kelly will go first.

ORAL ARGUMENT ON BEHALF OF THE JUNIOR PARTY

MR. O'CONNOR: Good morning. The subject matter of this interference involves a method for preventing or ameliorating premenstrual syndrome or symptoms associated with menopause. The method comprises administering to a woman an effective amount of isoflavones, compounds found in plants. They are part of a larger group of compounds known as phytoestrogens.

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Much of it goes to large discussion of how much extrinsic evidence you could turn to in construing and understanding that sentence, and it turns out nitroglycerine and scopolamine transdermal patches were commercially available.

When you say we can do it in the same manner you can do it with nitroglycerine and scopolamine. I think that's certainly a major difference. You don't need details in that sentence because the reference to nitroglycerin and scopolamine delivery provides those kind of differs.

In addition, that is strictly a 102 case. It doesn't deal at all with 103, and the issue is very different. In that case, it was only the delivery of nicotine. It had nothing to do with the effect the nicotine produced. That was indeed well-known. There were other ways, the nicotine gun was well-known. There are other ways of delivering nicotine to the body that were already known. It would be the case I think would only be analogous if it was previously established that the phytoestrogens were useful in treating menopause and the only issue was can you provide them in the form of a dietary supplement as opposed to as a food product. Then it becomes analogous.

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JUDGE McKELVEY: Why doesn't the Adlercreutz article?

MR. VOIGHT: That's not, I think, not at all I think the Adlercreutz article.

JUDGE McKELVEY: It says these folks eat more isoflavones than those folks and they seem to have less problems.

MR. VOIGHT: There are a lot of problems, I think, with the Adlercreutz article. Maybe the best place to start is claim construction. Let's construe some of these terms, because I think you do need to avoid the possibility of 102, construe the terms and what do they mean, and Judge McKelvey, have you identified in your order a bunch of terms that you wanted to discuss, including health supplement and extracted and naturally occurring and purified and concentrated.

The fact of the matter is all of these terms were used in an attempt to distinguish over food products. We are stuck with the English language and what you can say, but I think in, really in context of the Kelly specification, it's very clear that what he is talking about is not a food product. He is distinguishing over a food product and specifically I direct your attention to page 7 of

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the Kelly specification that's Gorbach Exhibit 1024.

JUDGE McKELVEY: This is a statement that you wouldn't expect people to change. You are going to come up with something, let them eat what they normally want to eat. These are my words now, and given this highly concentrated -- whatever.

MR. VOIGHT: After that, I don't need to read it, Judge McKelvey. That's exactly the gist of it. What they are saying is indeed it would be great if you could get people to change their diet, but you can't. So what's, down at the bottom then of page 7 he says an alternative strategy is to make available either phytoestrogens in purified form and that's one of our claims. We are using purified form or foodstuffs which are enriched. We didn't use the word enriched. That seems to be the term Mr. Eisenstein thinks we should have used. I wish we would have now.

It goes on to say in this way phytoestrogens.

JUDGE McKELVEY: He would just say if you mash these things and filter them out you have enriched it.

MR. VOIGHT: I think particularly the term that we use, health supplement which the sentence

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really gets into it, the last part at 7 and 8, says this way the phytoestrogen could be added to the diet in a convenient form as a supplement without requiring any substantive change in the diet, then the next sentence concerns a health supplement.

JUDGE McKELVEY: That takes care of Claim 21. What about 31 which doesn't seem to say anything about supplements?

MR. VOIGHT: It doesn't have that expression in there. Let me stick with dietary or health supplement. That is a well-known and defined term. Dietary supplement is defined in the FDA Act and as Mr. Eisenstein said, these are sold commercially. You are going to see lots of soy products, probably eight or ten different companies have soy products for treatment of menopause or other things on the market as dietary or health supplements, and it means something in addition to your diet. It doesn't mean food, and if you would like, I can refer you to how it's defined in the FDA Act.

In our Claims 21, 38, 34, 35, all of them use health supplement in terms of getting us where we want to go, I'll take any one of them. But again in context, why, why doesn't extracted, when you read

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it, I don't see how the whole context of the specification where it's clear that what we are trying to claim is something that is used to supplement the diet, but is not the actual diet itself, why extracted isn't such a term. But if we don't like extracted, we try these other expressions that our specification supports, like concentrated. And there was a lot of debate with Dr. Slavin about what concentrated or, that food products, it's not concentrated. And it's got to mean concentrated relative to the naturally occurring product.

Indeed, the claims use words like purified phytoestrogens extracted from soy or clover, or even extracted, they say extracted from soy or clover, isolated from naturally occurring, isolated. Again, clearly these terms are intended to mean removed from the, from the beans, and, or the clover, or whatever, whatever it comes from and that are, and then read in context for the whole specification, are used apart from the diet.

Now, Dr. Slavin, and I guess I'll, maybe I can point to some specific parts of that in a minute of her testimony, wants to take the position that it's purified or enriched or concentrated, whatever you want to say, even in food products from the

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beans. Well, that was typical of her testimony and why I think she is not entitled to any credibility. That's just not the case and she has got a publication she is an author on that shows that.

JUDGE McKELVEY: It may be the case. You start out with the soybean and you do something with it, you are going to concentrate the isoflavones and whatever it is you keep. Isn't it more of her problem that she really didn't address it in connection with contents of the specify case?

MR. VOIGHT: I think, Judge McKelvey, I agree with what you said, but also, though, if you will look at, this is Kelly Exhibit 2015, it's a publication that Slavin is an author of. And there is -- in her testimony, in discussing the issue of is it concentrated in food, I pointed out that she herself had a publication that contradicted her testimony, but that didn't stop her at all, she went right ahead and maintained that you get a higher concentration in food products.

But at table 2, for example, shows the concentration right at the top of table two, it's the third page of the publication of the first one, daidzein and then genistein and all three of those are isoflavones, the first three listed, and it shows

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first concentration in the soybean pieces, and then in three different food products. It's clear it goes down the amount of isoflavones present goes down. And after things like, well, particularly take miso soup. That's a very watery broth. Obviously the concentration is not very high in there.

And the text of this article makes reference to in processing the soybeans into food that you lose the isoflavone content. There is a couple of references, I won't read them, but the first full paragraph on the right-hand column on page 547 which is under the table we are looking at and then in the next page also the first full paragraph, last sentence and that's again in the right-hand column, there is specific references. So food products certainly are not and cannot be considered concentrated or purified.

JUDGE McKELVEY: You know, your Exhibit 2013, which is a consensus opinion, role of isoflavones, and so forth. Do you know if there was any cross-examination of her using that document?

MR. VOIGHT: There was cross-examination of her on this issue, and I made reference to her having a publication that was this publication that I had in mind, but at that point, I never placed it in

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front of her.

JUDGE McKELVEY: When you say this, 2015.

MR. VOIGHT: 2015. I never placed it in front of her at that time, but let's see, in her, must have, must have, I think, marked it at that, during that deposition, but I really -- I can't point to it right now.

JUDGE McKELVEY: It's kind of out of nowhere came that question. That's why sometimes it's nice to have a disk. Okay.

MR. VOIGHT: Right. I guess I want to again go back to dietary or health supplements and I guess I think I have made this point again. But they are, dietary supplements are defined in the Dietary Supplemental Health and Education Act which is the act that governs these exact products that are the actual commercial products made under these patents.

JUDGE McKELVEY: Do you have a cite for the act?

MR. VOIGHT: It's called the Dietary Supplemental Health and Education Act of 1994. What I have got which I would be happy to give you if you would like to receive it is a page off of the, just picked off of the FDA Internet site.

JUDGE McKELVEY: That's fine.

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MR. VOIGHT: That's just the first page, but that contains the definition right there of the dietary supplement, and I think it's clear that dietary supplement and health supplement are used interchangeably.

JUDGE McKELVEY: Now, that having been said, however, this is a specification that originated in Australia, and there is no indication in the spec itself that that's the definition Dr. Kelly wanted to use, so it would be true, wouldn't it, that we would look in the context of the specification.

MR. VOIGHT: There is certainly no reference in the specification to the FDA Act.

JUDGE McKELVEY: He is from Australia, so I don't know whether Australia has a similar act or not. But I mean this is more technical jargon, isn't it, than it would be legal definition?

MR. VOIGHT: I don't think that's so technical, actually. It says includes things like vitamins, minerals, herbs and candy extracts, our word, or concentrates and it may be in many forms such as tablets, capsules, soft gels, liquids or powders. Again, that's very consistent with what our specification says. And then it also makes the --

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JUDGE McKELVEY: So your argument would be that your specification is talking about the kinds of things the Dietary Supplemental Health and Education Act is talking about, even if it's not a one on one reference?

MR. VOIGHT: We are using it I think very consistent with the way the term dietary supplement or health supplement is used boy people active in this field.

JUDGE McKELVEY: Would you agree that the statement that in the specification, health supplement is used in a colloquial sense, it just happens to coincide with what the law --

MR. VOIGHT: Yes. Yes. That's true.

JUDGE McKELVEY: More or less.

MR. VOIGHT: I think the law is using it as it is normally used by those in this field and it's also used in the Kelly specification. I think at least some, if not all of our claims are not anticipated. I think that there is a limitation.

JUDGE McKELVEY: You think there might be one that is?

MR. VOIGHT: No. I really don't, because I think the term health supplement certainly is a key in our claim like 21. If there is one that is

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anticipated, it might indeed be, it might be indeed 31. If you don't -- if you read extracted as broadly as --

JUDGE McKELVEY: Dr. Slavin read it. In other words, if we agree with her on that interpretation, then maybe Claim 31 has a problem?

MR. VOIGHT: Exactly. But I also think if Claim 31 has got a problem, so does Gorbach's claim, which is identical. That's the claim copy, except for one word, extracted versus isolated.

JUDGE McKELVEY: Her theory is when you make the tofu you extract in the broadest sense.

MR. VOIGHT: And certainly you isolate it in the broadest sense also. So I don't see that there is any difference between their claims and our claims, which really I think is a reason to construe both claims to exclude food products. That's not what the, what is clear from the specification. Certainly it's clear from the Kelly specification. That's not what he is talking about.

JUDGE McKELVEY: Well, I mean it would be just as ridiculous to imagine that Gorbach is trying to claim a method of eating tofu would be that you are.

MR. VOIGHT: Absolutely.

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JUDGE McKELVEY: Because otherwise these claims don't make sense.

MR. VOIGHT: Absolutely. But I think it's sauce for the goose and sauce for the gander. I think it applies to both of them and neither of us I think wants the result that nobody gets a patent. That doesn't help.

JUDGE McKELVEY: That's not good for business.

MR. VOIGHT: It's not good for -- well, in the nature, we are a small company. We need the patent. They are a university. They are not making a product. They need the patent. We both want a patent here. It's not a position that either of us I think would find desirable.

Now, with regard, let me talk about obviousness a bit. I think from the questions that have come from the bench, it's clear that the panel is aware of the issue and the issues are is this Adlercreutz really a method to experiment or from Adlercreutz do you have a chance of success.

I'd like to start with the basic premise of Adlercreutz, that is that Japanese women have fewer menopausal symptoms. He relies for that premise on Lock, not on any actual study of his own

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U. S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
January 3, 2001

Overview of Dietary Supplements

What is a dietary supplement?

Congress defined the term "dietary supplement" in the Dietary Supplement Health and Education Act (DSHEA) of 1994. A dietary supplement is a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet. The "dietary ingredients" in these products may include: vitamins, minerals, herbs or other botanicals, amino acids, and substances such as enzymes, organ tissues, glandulars, and metabolites. Dietary supplements can also be extracts or concentrates, and may be found in many forms such as tablets, capsules, softgels, gelcaps, liquids, or powders. They can also be in other forms, such as a bar, but if they are, information on their label must not represent the product as a conventional food or a sole item of a meal or diet. Whatever their form may be, DSHEA places dietary supplements in a special category under the general umbrella of "foods," not drugs, and requires that every supplement be labeled a dietary supplement.

What is a "new dietary ingredient" in a dietary supplement?

The Dietary Supplement Health and Education Act (DSHEA) of 1994 defined both of the terms "dietary ingredient" and "new dietary ingredient" as components of dietary supplements. In order for an ingredient of a dietary supplement to be a "dietary ingredient," it must be one or any combination of the following substances:

- a vitamin,
- a mineral,
- an herb or other botanical,
- an amino acid,
- a dietary substance for use by man to supplement the diet by increasing the total dietary intake (e.g., enzymes or tissues from organs or glands), or
- a concentrate, metabolite, constituent or extract.

A "new dietary ingredient" is one that meets the above definition for a "dietary ingredient" and was not sold in the U.S. in a dietary supplement before October 15, 1994.

What is FDA's role in regulating dietary supplements versus the manufacturer's responsibility for marketing them?

In October 1994, the Dietary Supplement Health and Education Act (DSHEA) was signed into law by President Clinton. Before this time, dietary supplements were subject to the same regulatory requirements as were other foods. This new law, which amended the Federal Food, Drug, and Cosmetic Act, created a new regulatory framework for the safety and labeling of dietary supplements.

Under DSHEA, a firm is responsible for determining that the dietary supplements it manufactures or distributes are safe and that any representations or claims made about them are substantiated by adequate evidence to show that they are not false or misleading. This means that dietary supplements

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DIRECTIVE 2002/46/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL

of 10 June 2002

on the approximation of the laws of the Member States relating to food supplements

(Text with EEA relevance)

THE EUROPEAN PARLIAMENT AND THE COUNCIL OF THE EUROPEAN UNION,

Having regard to the Treaty establishing the European Community, and in particular Article 95 thereof,

Having regard to the proposal from the Commission (1),

Having regard to the opinion of the Economic and Social Committee (2),

Acting in accordance with the procedure laid down in Article 251 of the Treaty (3),

Whereas:

(1) There is an increasing number of products marketed in the Community as foods containing concentrated sources of nutrients and presented for supplementing the intake of those nutrients from the normal diet.

(2) Those products are regulated in Member States by differing national rules that may impede their free movement, create unequal conditions of competition, and thus have a direct impact on the functioning of the internal market. It is therefore necessary to adopt Community rules on those products marketed as foodstuffs.

(3) An adequate and varied diet could, under normal circumstances, provide all necessary nutrients for normal development and maintenance of a healthy life in quantities which meet those established and recommended by generally acceptable scientific data. However, surveys show that this ideal situation is not being achieved for all nutrients and by all groups of the population across the Community.

(4) Consumers, because of their particular lifestyles or for other reasons, may choose to supplement their intake of some nutrients through food supplements.

(5) In order to ensure a high level of protection for consumers and facilitate their choice, the products that will be put on to the market must be safe and bear adequate and appropriate labelling.

(6) There is a wide range of nutrients and other ingredients that might be present in food supplements including, but not limited to, vitamins, minerals, amino acids, essential fatty acids, fibre and various plants and herbal extracts.

(7) As a first stage, this Directive should lay down specific rules for vitamins and minerals used as ingredients of food supplements. Food supplements containing vitamins or minerals as well as other ingredients should also be in conformity with the specific rules on vitamins and minerals laid down in this Directive.

(8) Specific rules concerning nutrients, other than vitamins and minerals, or other substances with a nutritional or physiological effect used as ingredients of food supplements should be laid down at a later stage, provided that adequate and appropriate scientific data about them become available. Until such specific Community rules are adopted and without prejudice to the provisions of the Treaty, national rules concerning nutrients or other substances with nutritional or physiological effect used as ingredients of food supplements, for which no Community specific rules have been adopted, may be applicable.

(9) Only vitamins and minerals normally found in, and consumed as part of, the diet should be allowed to be present in food supplements although this does not mean that their presence therein is necessary. Controversy as to the identity of those nutrients that could potentially arise should be avoided. Therefore, it is appropriate to establish a positive list of those vitamins and minerals.

(10) There is a wide range of vitamin preparations and mineral substances used in the manufacture of food supplements currently marketed in some Member States that have not been evaluated by the Scientific Committee on Food and consequently are not included in the positive lists. These should be submitted to the European Food Safety Authority for urgent evaluation, as soon as appropriate files are presented by the interested parties.

(1) OJ C 311 E, 31.10.2000, p. 207 and C 180 E, 26.6.2001, p. 248.
(2) OJ C 14, 16.1.2001, p. 42.
(3) Opinion of the European Parliament of 14 February 2001 (OJ C 276, 1.10.2001, p. 126), Council Common Position of 3 December 2001 (OJ C 90 E, 16.4.2002, p. 1) and Decision of the European Parliament of 13 March 2002. Council Decision of 30 May 2002.

(11) The chemical substances used as sources of vitamins and minerals in the manufacture of food supplements should be safe and also be available to be used by the body. For this reason, a positive list of those substances should also be established. Such substances as have been approved by the Scientific Committee on Food, on the basis of the said criteria, for use in the manufacture of foods intended for infants and young children and other foods for particular nutritional uses can also be used in the manufacture of food supplements.

(12) In order to keep up with scientific and technological developments it is important to revise the lists promptly, when necessary. Such revisions would be implementing measures of a technical nature and their adoption should be entrusted to the Commission in order to simplify and expedite the procedure.

(13) Excessive intake of vitamins and minerals may result in adverse effects and therefore necessitate the setting of maximum safe levels for them in food supplements, as appropriate. Those levels must ensure that the normal use of the products under the instructions of use provided by the manufacturer will be safe for the consumer.

(14) When maximum levels are set, therefore, account should be taken of the upper safe levels of the vitamins and minerals, as established by scientific risk assessment based on generally acceptable scientific data, and of intakes of those nutrients from the normal diet. Due account should also be taken of reference intake amounts when setting maximum levels.

(15) Food supplements are purchased by consumers for supplementing intakes from the diet. In order to ensure that this aim is achieved, if vitamins and minerals are declared on the label of food supplements, they should be present in the product in a significant amount.

(16) The adoption of the specific values for maximum and minimum levels for vitamins and minerals present in food supplements, based on the criteria set out in this Directive and appropriate scientific advice, would be an implementing measure and should be entrusted to the Commission.

(17) General labelling provisions and definitions are contained in Directive 2000/13/EC of the European Parliament and of the Council of 20 March 2000 on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs [1], and do not need to be repeated. This Directive should therefore be confined to the necessary additional provisions.

(18) Council Directive 90/496/EEC of 24 September 1990 on nutrition labelling for foodstuffs [2] does not apply to food supplements. Information relating to nutrient content in food supplements is essential for allowing the consumer who purchases them to make an informed choice and use them properly and safely. That information should, in view of the nature of those products, be confined to the nutrients actually present and be compulsory.

(19) Given the particular nature of food supplements, additional means to those usually available to monitoring bodies should be available in order to facilitate efficient monitoring of those products.

(20) The measures necessary for the implementation of this Directive should be adopted in accordance with Council Decision 1999/468/EC of 28 June 1999 laying down the procedures for the exercise of implementing powers conferred on the Commission [3],

HAVE ADOPTED THIS DIRECTIVE:

Article 1

1. This Directive concerns food supplements marketed as foodstuffs and presented as such. These products shall be delivered to the ultimate consumer only in a pre-packaged form.

2. This Directive shall not apply to medicinal products as defined by Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use [4].

Article 2

For the purposes of this Directive:

(a) 'food supplements' means foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities;

(b) 'nutrients' means the following substances:

(i) vitamins,

(ii) minerals.

[1] OJ L 109, 6.5.2000, p. 29.

[2] OJ L 276, 6.10.1990, p. 40.
[3] OJ L 184, 17.7.1999, p. 23.
[4] OJ L 311, 28.11.2001, p. 67.

Article 3

Member States shall ensure that food supplements may be marketed within the Community only if they comply with the rules laid down in this Directive.

Article 4

1. Only vitamins and minerals listed in Annex I, in the forms listed in Annex II, may be used for the manufacture of food supplements, subject to paragraph 6.

2. The purity criteria for substances listed in Annex II shall be adopted in accordance with the procedure referred to in Article 13(2), except where they apply pursuant to paragraph 3.

3. Purity criteria for substances listed in Annex II, specified by Community legislation for their use in the manufacture of foodstuffs for purposes other than those covered by this Directive, shall apply.

4. For those substances listed in Annex II for which purity criteria are not specified by Community legislation, and until such specifications are adopted, generally acceptable purity criteria recommended by international bodies shall be applicable and national rules setting stricter purity criteria may be maintained.

5. Modifications to the lists referred to in paragraph 1 shall be adopted in accordance with the procedure referred to in Article 13(2).

6. By way of derogation from paragraph 1 and until 31 December 2009, Member States may allow in their territory the use of vitamins and minerals not listed in Annex I, or in forms not listed in Annex II, provided that:

(a) the substance in question is used in one or more food supplements marketed in the Community on the date of entry into force of this Directive,

(b) the European Food Safety Authority has not given an unfavourable opinion in respect of the use of that substance, or its use in that form, in the manufacture of food supplements, on the basis of a dossier supporting use of the substance in question to be submitted to the Commission by the Member State not later than 12 July 2005.

7. Notwithstanding paragraph 6, Member States may, in compliance with the rules of the Treaty, continue to apply existing national restrictions or bans on trade in food supplements containing vitamins and minerals not included in the list in Annex I or in the forms not listed in Annex II.

8. Not later than 12 July 2007, the Commission shall submit to the European Parliament and the Council a report on the advisability of establishing specific rules, including, where appropriate, positive lists, on categories of nutrients or of substances with a nutritional or physiological effect other than those referred to in paragraph 1, accompanied by any proposals for amendment to this Directive which the Commission deems necessary.

Article 5

1. Maximum amounts of vitamins and minerals present in food supplements per daily portion of consumption as recommended by the manufacturer shall be set, taking the following into account:

(a) upper safe levels of vitamins and minerals established by scientific risk assessment based on generally accepted scientific data, taking into account, as appropriate, the varying degrees of sensitivity of different consumer groups;

(b) intake of vitamins and minerals from other dietary sources.

2. When the maximum levels referred to in paragraph 1 are set, due account should also be taken of reference intakes of vitamins and minerals for the population.

3. To ensure that significant amounts of vitamins and minerals are present in food supplements, minimum amounts per daily portion of consumption as recommended by the manufacturer shall be set, as appropriate.

4. The maximum and minimum amounts of vitamins and minerals referred to in paragraphs 1, 2 and 3 shall be adopted in accordance with the procedure referred to in Article 13(2).

Article 6

1. For the purposes of Article 5(1) of Directive 2000/13/EC, the name under which products covered by this Directive are sold shall be 'food supplement'.

2. The labelling, presentation and advertising must not attribute to food supplements the property of preventing, treating or curing a human disease, or refer to such properties.

3. Without prejudice to Directive 2000/13/EC, the labelling shall bear the following particulars:

(a) the names of the categories of nutrients or substances that characterise the product or an indication of the nature of those nutrients or substances;

(b) the portion of the product recommended for daily consumption;

(c) a warning not to exceed the stated recommended daily dose;

(d) a statement to the effect that food supplements should not be used as a substitute for a varied diet;

(e) a statement to the effect that the products should be stored out of the reach of young children.

Article 7

The labelling, presentation and advertising of food supplements shall not include any mention stating or implying that a balanced and varied diet cannot provide appropriate quantities of nutrients in general.

Rules for implementing this Article may be specified in accordance with the procedure referred to in Article 13(2).

Article 8

1. The amount of the nutrients or substances with a nutritional or physiological effect present in the product shall be declared on the labelling in numerical form. The units to be used for vitamins and minerals shall be those specified in Annex I.

Rules for implementing this paragraph may be specified in accordance with the procedure referred to in Article 13(2).

2. The amounts of the nutrients or other substances declared shall be those per portion of the product as recommended for daily consumption on the labelling.

3. Information on vitamins and minerals shall also be expressed as a percentage of the reference values mentioned, as the case may be, in the Annex to Directive 90/496/EEC.

Article 9

1. The declared values mentioned in Article 8(1) and (2) shall be average values based on the manufacturer's analysis of the product.

Further rules for implementing this paragraph with regard in particular to the differences between the declared values and those established in the course of official checks shall be decided upon in accordance with the procedure referred to in Article 13(2).

2. The percentage of the reference values for vitamins and minerals mentioned in Article 8(3) may also be given in graphical form.

Rules for implementing this paragraph may be adopted in accordance with the procedure referred to in Article 13(2).

Article 10

To facilitate efficient monitoring of food supplements, Member States may require the manufacturer or the person placing the product on the market in their territory to notify the competent authority of that placing on the market by forwarding it a model of the label used for the product.

Article 11

1. Without prejudice to Article 4(7), Member States shall not, for reasons related to their composition, manufacturing specifications, presentation or labelling, prohibit or restrict trade in products referred to in Article 1 which comply with this Directive and, where appropriate, with Community acts adopted in implementation of this Directive.

2. Without prejudice to the Treaty, in particular Articles 28 and 30 thereof, paragraph 1 shall not affect national provisions which are applicable in the absence of Community acts adopted under this Directive.

Article 12

1. Where a Member State, as a result of new information or of a reassessment of existing information made since this Directive or one of the implementing Community acts was adopted, has detailed grounds for establishing that a product referred to in Article 1 endangers human health though it complies with the said Directive or said acts, that Member State may temporarily suspend or restrict application of the provisions in question within its territory. It shall immediately inform the other Member States and the Commission thereof and give reasons for its decision.

2. The Commission shall examine as soon as possible the grounds adduced by the Member State concerned and shall consult the Member States within the Standing Committee on the Food Chain and Animal Health, and shall then deliver its opinion without delay and take appropriate measures.

3. If the Commission considers that amendments to this Directive or to the implementing Community acts are necessary in order to remedy the difficulties mentioned in paragraph 1 and to ensure the protection of human health, it shall initiate the procedure referred to in Article 13(2) with a view to adopting those amendments. The Member State that has adopted safeguard measures may in that event retain them until the amendments have been adopted.

Article 13

1. The Commission shall be assisted by the Standing Committee on the Food Chain and Animal Health instituted by Regulation (EC) No 178/2002 (1) (hereinafter referred to as 'the Committee').

2. Where reference is made to this paragraph, Articles 5 and 7 of Decision 1999/468/EC shall apply, having regard to the provisions of Article 8 thereof.

The period laid down in Article 5(6) of Decision 1999/468/EC shall be set at three months.

3. The Committee shall adopt its rules of procedure.

(1) OJ L 31, 1.2.2002, p. 1.

Article 14

Provisions that may have an effect upon public health shall be adopted after consultation with the European Food Safety Authority.

Article 15

Member States shall bring into force the laws, regulations and administrative provisions necessary to comply with this Directive by 31 July 2003. They shall forthwith inform the Commission thereof.

Those laws, regulations and administrative provisions shall be applied in such a way as to:

(a) permit trade in products complying with this Directive, from 1 August 2003 at the latest;

(b) prohibit trade in products which do not comply with the Directive, from 1 August 2005 at the latest.

When Member States adopt these measures, they shall contain a reference to this Directive or be accompanied by such a reference on the occasion of their official publication. The methods of making such reference shall be adopted by the Member States.

Article 16

This Directive shall enter into force on the day of its publication in the *Official Journal of the European Communities*.

Article 17

This Directive is addressed to the Member States.

Done at Luxembourg, 10 June 2002.

For the European Parliament	*For the Council*
The President	*The President*
P. COX	J. PIQUÉ I CAMPS

ANNEX I

Vitamins and minerals which may be used in the manufacture of food supplements

1. Vitamins

Vitamin A (µg RE)
Vitamin D (µg)
Vitamin E (mg α-TE)
Vitamin K (µg)
Vitamin B1 (mg)
Vitamin B2 (mg)
Niacin (mg NE)
Pantothenic acid (mg)
Vitamin B6 (mg)
Folic acid (µg)
Vitamin B12 (µg)
Biotin (µg)
Vitamin C (mg)

2. Minerals

Calcium (mg)
Magnesium (mg)
Iron (mg)
Copper (µg)
Iodine (µg)
Zinc (mg)
Manganese (mg)
Sodium (mg)
Potassium (mg)
Selenium (µg)
Chromium (µg)
Molybdenum (µg)
Fluoride (mg)
Chloride (mg)
Phosphorus (mg)

ANNEX II

Vitamin and mineral substances which may be used in the manufacture of food supplements

A. Vitamins

1. VITAMIN A
   (a) retinol
   (b) retinyl acetate
   (c) retinyl palmitate
   (d) beta-carotene

2. VITAMIN D
   (a) cholecalciferol
   (b) ergocalciferol

3. VITAMIN E
   (a) D-alpha-tocopherol
   (b) DL-alpha-tocopherol
   (c) D-alpha-tocopheryl acetate
   (d) DL-alpha-tocopheryl acetate
   (e) D-alpha-tocopheryl acid succinate

4. VITAMIN K
   (a) phylloquinone (phytomenadione)

5. VITAMIN B1
   (a) thiamin hydrochloride
   (b) thiamin mononitrate

6. VITAMIN B2
   (a) riboflavin
   (b) riboflavin 5'-phosphate, sodium

7. NIACIN
   (a) nicotinic acid
   (b) nicotinamide

8. PANTOTHENIC ACID
   (a) D-pantothenate, calcium
   (b) D-pantothenate, sodium
   (c) dexpanthenol

9. VITAMIN B6
   (a) pyridoxine hydrochloride
   (b) pyridoxine 5'-phosphate

10. FOLIC ACID
   (a) pteroylmonoglutamic acid

11. VITAMIN B12
   (a) cyanocobalamin
   (b) hydroxocobalamin

12. BIOTIN
   (a) D-biotin

13. VITAMIN C
   (a) L-ascorbic acid
   (b) sodium-L-ascorbate
   (c) calcium-L-ascorbate
   (d) potassium-L-ascorbate
   (e) L-ascorbyl 6-palmitate

B. Minerals

calcium carbonate
calcium chloride
calcium salts of citric acid
calcium gluconate
calcium glycerophosphate
calcium lactate
calcium salts of orthophosphoric acid
calcium hydroxide
calcium oxide
magnesium acetate
magnesium carbonate
magnesium chloride
magnesium salts of citric acid
magnesium gluconate
magnesium glycerophosphate
magnesium salts of orthophosphoric acid
magnesium lactate
magnesium hydroxide
magnesium oxide
magnesium sulphate
ferrous carbonate
ferrous citrate
ferric ammonium citrate
ferrous gluconate
ferrous fumarate
ferric sodium diphosphate
ferrous lactate
ferrous sulphate
ferric diphosphate (ferric pyrophosphate)
ferric saccharate
elemental iron (carbonyl+electrolytic+hydrogen reduced)
cupric carbonate
cupric citrate
cupric gluconate
cupric sulphate
copper lysine complex

sodium iodide
sodium iodate
potassium iodide
potassium iodate
zinc acetate
zinc chloride
zinc citrate
zinc gluconate
zinc lactate
zinc oxide
zinc carbonate
zinc sulphate
manganese carbonate
manganese chloride
manganese citrate
manganese gluconate
manganese glycerophosphate
manganese sulphate
sodium bicarbonate
sodium carbonate
sodium chloride
sodium citrate
sodium gluconate
sodium lactate
sodium hydroxide
sodium salts of orthophosphoric acid
potassium bicarbonate
potassium carbonate
potassium chloride
potassium citrate
potassium gluconate
potassium glycerophosphate
potassium lactate
potassium hydroxide
potassium salts of orthophosphoric acid
sodium selenate
sodium hydrogen selenite
sodium selenite
chromium (III) chloride
chromium (III) sulphate
ammonium molybdate (molybdenum (VI))
sodium molybdate (molybdenum (VI))
potassium fluoride
sodium fluoride

D20

MEDICAMENTS ~~HEALTH SUPPLEMENTS~~ CONTAINING PHYTO-OESTROGENS, ANALOGUES OR METABOLITES ~~THEREOF~~ AND USES THEREOF

TECHNICAL FIELD

This invention relates to ~~natural products~~ medicaments containing phyto-oestrogens, or phyto-oestrogen metabolites used to treat pre-menstrual syndrome, menopausal symptoms, or prostate cancer. ~~which have various beneficial physiological effects in man, and which have a variety of uses, such as to promote good health and as a dietary additive, for example~~.

BACKGROUND ART

The ~~particular product~~ medicaments for use in accordance with the invention ~~is of~~ are made from extracts of certain plants with the particular purpose of enrichment for phyto-oestrogens, both in their natural state and their closely related derivatives and metabolites.

Plants which are used as foodstuffs or medicinal herbs contain a wide variety of chemicals which are assimilated into the body following ingestion. Some of these chemicals are important nutrients for man and animals (e.g. fats, carbohydrates, proteins, vitamins, minerals) while others have none, or little or no known nutritional value. The phyto-oestrogens hitherto have fallen into this latter category of no known nutritional value.

There are 3 principal classes of phyto-oestrogens, viz. isoflavones, lignans, and coumestans. The isoflavones are thought to have a broad range of biological functions in plants, although these are poorly understood. However, two particular functions are recognised - (a) as phyto-alexin or stressor chemicals which are secreted by the plant in response to attack by parasites such as insects, fungi, viruses, etc and which display activity against these parasites, and (b) chemicals which encourage colonisation of nitrogen-fixing bacteria on the roots of legumes. The biological functions in plants of the lignans and coumestans is not generally understood.

The different types of phyto-oestrogens are as follows.

Type 1 phyto-oestrogens - (isoflavones)

Isoflavones appear to be widely distributed in the plant kingdom and over 700 different isoflavones are described. However, the isoflavones which display oestrogenic activity belong to a small sub-group and are restricted almost exclusively to the *Leguminosae* family. The known oestrogenic isoflavones are daidzein, formononetin, genistein and biochanin A. In

(AUXILIARY REQUEST)

be added to the diet in a convenient form as a supplement without requiring any substantive change to the diet.

The present invention provides the use of an isoflavone phyto-oestrogen extract of soy or clover for the manufacture of a medicament for administration in unit dosage form for the treatment of pre-menstrual syndrome, symptoms associated with the menopause, or prostate cancer.

The medicament may further comprise at least one dietary suitable excipient, preferably wherein the medicament is in the form of a tablet or capsule.

The isoflavone phyto-estrogens are preferably present in an amount of from about 20mg to 200mg per dosage unit, optionally wherein the amount is 50mg to 150mg.

In preferred embodiments the extract is obtained from clover or soybean. The extract is most preferably obtained from the leaves of clover or the hypocotyls of soybean.

The extract is preferably in liquid form, more preferably an aqueous organic solvent extract, even more preferably an aqueous alcohol extract.

In preferred embodiments the administration of the medicament is at least daily over a period of at least a month. ~~Additionally, the invention provides the use of isolated soybean hypocotyls, optionally ground or milled, as a human dietary additive or supplement.~~

~~In another aspect the invention also provides isolated soybean hypocotyls, optionally ground or milled, for use as a pharmaceutical for oral administration.~~

~~Thus the invention provides the use of isolated soybean hypocotyls, optionally ground or milled, for the manufacture of a medicament for oral administration for the treatment of pre-menstrual syndrome, symptoms associated with menopause, elevated levels of cholesterol in the blood stream, or prostate cancer.~~

~~In either of the aspects of the invention described above administration of the medicament is preferably at least daily over a period of at least a month.~~

The medicaments of the present invention ~~concerns a health supplement~~ are specifically enriched for isoflavones selected from genistein, daidzein, formononetin and biochanin A, or their natural glycoside form, or their analogues, ~~in sufficient amounts to improve the health of a human.~~

Preferably the ~~supplement~~ medicament contains an excipient, a diluent, a carrier or the like, or else ~~the supplement~~ it may be is mixed with food or can be consumed directly. ~~It is also preferred that foodstuffs, are readily available, have no known toxic components, and are rich sources of isoflavones; such foodstuffs preferably being red clover or soya.~~ It is also preferred that the ratio of genistein and/or it methylated derivative biochanin A to daidzein and/or its methylated derivative formononetin is between 1:2 to 2:1. Other plant components with oestrogenic activity including lignans, coumestans and flavones may also be present in extract, but it is held that these are of secondary importance to the predominant isoflavones. The term phyto-estrogens is used hereafter to indicate a predominance of isoflavones with lesser amounts of lignans, coumestans and flavones.

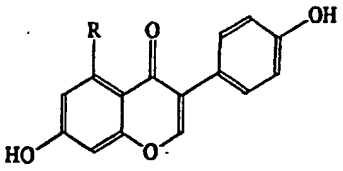
medicaments in accordance with the ... can be used to improve

The invention ~~also concerns a method of improving~~ the health of a human by administering to the human ~~a sufficient amount of phyto-oestrogen. Ideally, the phyto-oestrogen is administered~~ regularly on a daily basis over a sufficient period such as at least a month. The health conditions which ~~may be~~ are prevented or ameliorated include ~~cancer of the breast,~~ cancer of the prostate, ~~cancer of the uterus, cancer of the bowel, benign (or cystic) breast disease,~~ pre-menstrual syndrome (also known as pre-menstrual tension), or adverse symptoms associated with menopause in women. ~~The method and supplement in accordance with the invention also improves the health of a human having elevated levels of blood cholesterol. The product also is useful in avoiding or ameliorating cancer in persons. The symptoms produced by these conditions and the general well-being is also improved by the use of these supplements.~~

~~The phyto-oestrogen in accordance with the invention may be obtained from a number of different sources. Preferably~~ The phyto-oestrogens are extracted from a clover, such as red clover or subterranean clover, or from soya which contain high levels of phyto-oestrogens.

~~However, any source rich in phyto-oestrogens may be used instead, if desired.~~

Various different isoflavones have been identified from these sources - they are principally genistein, biochanin A, daidzein, formononetin and glycitein. In plants these compounds occur principally in a glycoside form bound to sugars such as glucose, with smaller amounts present as the aglucone forms. The formulae of the isoflavones are:



daidzein R = H
genistein R = OH

The structure of biochanin A is the same as for genistein but with a 4'-methoxy group, and similarly formononetin has the same structure as daidzein, but with a 4'-methoxy group.

Following ingestion by humans, the glycosidic isoflavones are hydrolysed to the aglucone form and biochanin A and formononetin are demethylated by bacterial fermentation to genistein and daidzein respectively. A small proportion of these free isoflavones are absorbed directly from the bowel and circulate in the blood. The bulk of the isoflavones, however, remain in the bowel and undergo fermentation to form various metabolites which also are absorbed into the bloodstream. The principal metabolites which have been identified are equol and O-*des*methylangolensin.

In vitro and *in vivo* studies have indicated that genistein, biochanin A, equol, daidzein, formononetin all have oestrogenic activity in descending order. O-*des*methylangolensin is only very weakly oestrogenic and glycitein is non-oestrogenic.

In animal and *in vitro* studies, genistein has been shown to have greater oestrogenic/anti-oestrogenic activity and SHBG-stimulating capacity than the other isoflavones or their metabolites (approximately 10 times that of daidzein and formononetin). However, the full range of biological effects of the different isoflavones have yet to be fully determined, and in particular their relative efficacies in the different biological effects such as oestrogenicity,

hypocholesterolaemia, anti-angiogenesis, anti-oxidation, anti-carcinogenesis for example are not yet fully known.

It is thought that because the methyl forms (biochanin A and formononetin) ultimately are largely demethylated to their principals, genistein and daidzein, with improved biological efficacy, then it is unimportant whether the isoflavones are present in the claimed ~~product~~ in the methylated or demethylated forms.

Given that the relative biological importance of the two isoflavone groups (being genistein and daidzein) to human health remains unclear, and that each might indeed have different importance, plus the fact that both isoflavones are present in the diet in approximately equal proportions, then it is prudent that both isoflavones be present in the claimed ~~product~~ in approximately equal proportions.

~~Any leguminous plants such as detailed here could be used as sources of phyto-oestrogens~~ (principally isoflavones with lesser amounts of lignans and coumestans): Indian liquorice (*Abrus precatorius*); various species of *Acacia spp.* including, *A. aneura*, *A. cibaria*, *A. longifolia*, and *A. oswaldii*; ground nut (*Apio tuberosa*); ground pea (*Arachis hypogea*); milk vetch (*Astragalus edulis*); marama bean (*Bauhinia esculenta*); sword bean (*Cajanus cajan indicus*); jack bean (*Canavalia ensiformis*); sword bean (*Canavalia gladiata*); seaside sword bean (*Canavalia rosea*); various *Cassia spp.* including *C. floribunda*, *C. laevigata*, and *C. occidentalis*; carobbean (*Ceratonia siliqua*); chick pea (*Cicer arietinum*); yebnut (*Cordeauxia edulis*); various *Crotalaria spp.* including *C. laburnifolia*, and *C. pallida*; cluster bean (*Cyamopsis psoralioides*); tallow tree (*Detariaum senegalense*); sword bean (*Entada scandens*); balu (*Erythrina edulis*); soyabean (*Clycine max*;) inga (*Ingaedulis*); Polynesian chestnut (*Inocarpus fagifer*); hyacinth bean (*Lablab purpureus*); grass pea or Indian vetch (*Lathyrus sativus*); cyprus vetch (*Lathyrus ochrus*); lentil (*Lens culinaris*); jumping bean (*Leucaenal eucocephala*); various *Lupinus spp.* including *L. albus*, *L. luteus*, *L. angustifolium*, *L. mutabilis*, and *L. cosentinii*; ground bean (*Macotylma geocarpa*); horse gram (*Macrotyloma uniflorum*); alfalfa (*Medicago sativa*); velvet bean (*Mucuna pruriens*); yam beans (*Pachyrhyzus erosus*, *P. tuberosus*); African locust bean (*Parkia clappertoniana*); *Parkia speciosa*; oil bean tree (*Pentaclethra macrophylla*); various *Phaseolus spp.* including *P. acutifolius*, *P. vulgaris*, ~~*P. lunatus*, *P. coccineus*, *P. adenathus*, *P. angularis*, *P. aureus*, *P. calcaratus*, *P. mungo*, and *P.*~~

~~polystachyus; garden pea (*Pisum sativum*); djenko bean (*Pithecolobium lobatum*); mesquite~~ (various *Prosopis spp.*); goa bean (*Psophocarpus scandens, P. tetragonolobus*); ~~various~~ *Psoralea spp.*; *Sesbania bispinosa*; yam bean (*Sphenostylis stenocarpa*); ~~tamarind~~ (*Tamarindus indica*); fenugreek (*Trigonella foenum-graecum*); ~~vetches~~ (various *Vivia spp.* including *V. sativa, V. atropurpurea, V. ervilia,* and ~~*V. monantha*~~); broad bean (*Vicia faba*); black gram (*Vigna mungo*); ~~various *Vigna spp.*~~ including *V. radiata, V. aconitifolia, V. adanatha, V. angularus, V. trilobata, V. umbelata,* ~~and *V. unguiculata,* and, earth pea (*Voandzeia subterranea*).~~

soy or clover

The ideal sources of phyto-oestrogens ~~for preparation of a supplement~~ in accordance with the invention are preferably those which (i) are readily available, (ii) are relatively inexpensive, (iii) are readily and economically processed so as to yield the extract, (iv) have a high isoflavone content so as to provide high yields, and (v) have no known toxic components requiring selective removal or inactivation.

Certain clovers, such as red clover (*T. pratense*) and subterranean clover (*T. subterranean*) are the preferred sources. On a dry weight basis, these clovers contain the highest amounts of oestrogenic isoflavones of all legumes tested to date with levels of 3-5 g% (*T. subterranean*) and 1-3 g% (*T. pratense*). In comparison, soya flour has a level of 0.15-0.30 g%, lentils (0.08-0.12 g%), chick peas (0.07-0.13 g%), and garden peas (0.02-0.03 g%). Thus it can be seen that clovers contain approximately at least 10-30 times by weight the isoflavone content of other commonly available, human leguminous foodstuffs meaning that for manufacturing purposes, the yield of isoflavones per unit weight of plant material is many times greater from clover than from other legumes.

Red clover and subterranean clover also are common fodder crops and are readily grown and are widely available. Clovers also are comparatively cheaper ($200/tonne) than crops such as soya and lentils ($500/tonne).

With clovers, the isoflavones are recovered from the leaf rather than from the seed in the case of soya, beans, nuts and grams. This provides a substantially higher yield of isoflavones per unit area of pasture for clovers compared to other legumes because of the greater leaf matter compared to seed matter recovered per plant.

Clovers also have an extended growing season, and faster growth rates compared to those legumes such as soya, lentils or chick peas where the seed is the end-product. Clover can be cropped for its leaf content repeatedly over a single growing season. An additional benefit of this is that as phyto-alexins, the isoflavone content increases in response to the stress of cropping.

Thus it can be seen that in clovers versus other legumes provide a combination of (a) higher isoflavone content per dry weight of plant, (b) a higher yield of dry matter containing isoflavones per plant, and (c) a higher yield of dry matter per hectare.

An additional feature of clovers is that there are wide varieties of cultivars with widely differing isoflavone levels and types. This allows blending of different cultivars to achieve the desired ratio of the different isoflavones, although it is equally possible to use a single cultivar which provides the desired ratio.

~~Other legumes such as~~ Soyabean flour may be used as the source ~~for enrichment~~ of phyto-oestrogens but the substantially poorer (approx. 10%) yield of isoflavones compared to clovers means that the manufacturing costs are substantially greater and there is substantially greater amounts of waste products which requires disposal or further treatment for re-use as a foodstuff. An alternative, however, to the use of whole soya for this purpose, is to use the hull and hypocotyl (or germ) of the whole soyabean. The hull and hypocotyl represent only a small proportion by weight (8% and 2% respectively) of the intact bean. However, the coumestrol content of soya is concentrated in the hull, and the daidzein content of soya is concentrated in the hypocotyl. The two cotyledons which comprise the bulk of the soyabean (90% by weight) contain the bulk of the genistein content of soya. During standard processing of soyabeans, the hulls being a fibrous component with little or no perceived nutritional value normally are separated and removed by physical means. The hypocotyls become separated following the splitting of the cotyledons, and while these currently generally are not deliberately isolated, they may be separated and isolated by passing the disturbed soyabeans over a sieve of sufficient pore size to selectively remove the small hypocotyl. The hypocotyl contains approx. 1.0-1.5 g% isoflavones (95% daidzein, 5% genistein). The raw hypocotyl and hull material can be ground or milled to produce, for example, a dry powder or flour which then could be either blended or used separately as a dietary supplement in a variety of

ways including, for example, as a powder, in a liquid form, in a granulated form, in a tablet or encapsulated form, or added to other prepared foodstuffs. ~~Alternatively~~ it ~~could be~~ further processed to yield an enriched extract of phyto-oestrogens. ~~Either or both of these~~ material also could be added to ~~other leguminous material such as~~ clover ~~to provide~~ the invention.

In plants, the oestrogenic isoflavones are restricted principally to the leaf, fruit and root; the stem and petiole contain very little. With soya ~~and other common human legume foodstuff~~ crops, the leaves are rarely regarded as foodstuff; indeed with these crops, the plants normally are allowed to die and dry out before the seed crop is harvested. Nevertheless, the fresh leaves of these crops could be regarded as a source of phyto-oestrogens for the invention although the much lower isoflavone content of the leaves of these crops compared to clovers, plus their generally slow growth compared to clovers, suggests that they would not be a preferred source of large-scale isoflavone enrichment.

To provide a similar amount of isoflavone to that contained in most traditional legume-rich diets (50-100 mg oestrogenic isoflavones/day) would require an average daily consumption of 3-6 g dry weight or 15-30 g wet weight of specially selected cultivars of clover with particularly high isoflavone levels. Clover grasses generally are not eaten by humans, except to a limited extent as sprouts of some of the pleasanter tasting varieties. Isoflavones are intensely astringent and are responsible in large part for the bitter taste of legumes. Thus the types of bean sprouts, clover sprouts and alfalfa sprouts generally available have been selected on the basis of cultivar and of age for pleasant taste, and in so doing inadvertently have been selected for low isoflavone content. Of the sprouts currently available in Western countries for human consumption, between approx. 100-250 g would need to be consumed daily to provide a dosage of 50-100 mg isoflavones. Certainly clovers and other legume sprouts are not generally eaten in such sufficient quantities by humans to obtain the advantages of the present invention.

The invention also concerns formulations containing the phyto-oestrogens discussed above together with a dietary suitable excipient, diluent, carrier, or with a food. Ideally the formulation is in the form of a pill, tablet, capsule, or similar dosage form.

The ~~formulations~~ may be ~~a variety of kinds, such~~ as nutritional supplements, pharmaceutical

preparations, vitamin supplements, food additives or foods supplemented with the specified active phyto-oestrogens of the invention, liquid or solid preparations, including drinks, sterile injectable solutions, tablets, coated tablets, capsules, powders, drops, suspensions, or syrups, ointments, lotions, creams, pastes, gels, or the like. The formulations may be in convenient dosage forms, and may also include other active ingredients, and/or may contain conventional excipients, carriers and diluents. The ~~inclusion of the subject phyto-oestrogens in~~ medicaments are preferably presented as herbal remedies and treatments ~~is also a preferred part of the invention.~~

The invention is ~~also~~ directed to the amelioration, prevention, or of ~~various~~ conditions ~~responsive to treatment with~~ using the phyto-oestrogen ~~substances of the invention~~ specified medicaments. The preferred amounts to be administered to the human fall within 20 - 200 mg on a daily basis. More preferably the dosage is from 50 - 150 mg on a daily basis, and most preferably at a dosage of about 100 mg. If desired greater dosages can be administered for therapeutic reasons. In contrast to prior practices such high dosages were not possible. For example, dosages of up to or greater than 1000 mg may be suitable ~~to treat some conditions~~. In order to obtain the benefits of the invention, the treatment with the isoflavones should continue for a considerable period, ideally for at least a month, and ideally continuously for the whole period for which the health improvement advantages should accrue.

The ~~product~~ medicament according to the claimed use of the present invention yields a constant and accurately known amount of isoflavones. The product is also ideally a natural product, which has advantages for consumer acceptance, and in accordance with the supposed theory behind the invention may very possibly be one of the main causes for its beneficial effects. Whole legumes have a widely variable isoflavone content due to two main causes: the type of legume and the environmental effect. The type of legume typically has a wide range of isoflavone content. The milligram of isoflavone per hundred gram of whole foodstuff (dry weight) is given in the following table:

Soya Products	
- Whole Soya	150 - 300
- Soya Milk	25 - 40 (mg per 200 ml)
- Tofu	55 - 95
Lentils	80 - 120
Chickpeas	70 - 130
Broad beans	15 - 20
Garden peas	15 - 25

Thus common leguminous foodstuffs consumed in Western countries (broad beans, garden peas etc) have relatively low oestrogenic isoflavone content and exceptionally large amounts of these would need to be consumed daily to approximate those isoflavone levels consumed in traditional diets. Most Western cultures do not traditionally eat legumes with high isoflavone contents, and those soya products (milk, tofu etc) which are becoming increasingly popular in Western countries, also have relatively low isoflavone levels compared to whole soya, indicating that relatively large amounts of these would need to be consumed on a regular basis to deliver the required isoflavone levels.

The enviromental effect arises because the isoflavone levels in any species of plant depend greatly on the age of the plant, the climatic conditions where it is grown, the fertiliser and so forth. Therefore constant and consistant dosage is very difficult with ordinary whole foodstuffs. The accurately determined quality and quantity of the active isoflavones in the ~~product~~ medicament, and its easy consumability when compared with the almost impossible task of eating huge amounts of often practically inedible foods, is therefore an import feature of the invention for preventing and helping in overcoming ~~various~~ the specified health problems.

~~Among~~ include the ~~various~~ health problems, the treatment or prevention of ~~high blood cholesterol levels,~~ and ~~the treatment of~~ PMS and menopausal symptoms ~~is especially important.~~

The ~~product~~ Medicament of the invention modulates the production and/or function of endogenous sex hormones in humans to modify or produce health improving effects, including the following: ~~(i) lowered levels of various blood lipoproteins including, for instance, low density and very low density cholesterol leading to reduced risk of development of atherosclerosis;~~ (ii) reduced risk of development of cancer of the prostate; ~~(iii) reduced risk of cancer of the~~

~~breast; (iv) reduced risk of development of cancer of the uterus; (v) reduced risk of development of cancer of the large bowel;~~ (vi) reduced risk of development of the syndrome in women commonly referred to pre-menstrual syndrome (PMS), which includes pre-menstrual tension (PMT); (vii) reduced risk of development of many untoward symptoms (including dry vagina, peripheral flushing, depression etc) commonly associated in women with menopause; ~~and (vi) treating benign breast disease in women (benign or cystic breast disease associated with non-malignant swelling and tenderness of breast tissue).~~ The invention therefore is directed to ~~a method~~ the use of the specified medicament for the prophylaxis or treatment of a human, to combat the specified conditions associated with phyto-oestrogen deficiency, which comprises administering to the human an effective amount of phyto-oestrogen principally isoflavone but which might also include relatively smaller amounts of lignans and coumestans, ideally in a concentrated form, wherein the isoflavones include genistein, and/or biochanin A, and/or daidzein, and/or formononetin.

~~Cancer of the breast generally is considered to be associated with oestrogenic dysfunction.~~ Breast cancer cells may display more oestrogen receptors than normal breast cells and stimulation of these receptors by endogenous oestrogens is thought to be a prime source of stimulation of their malignant growth. Currently synthetic anti-oestrogens are being used to prevent or treat the growth of malignant breast cells. Isoflavones are potent anti-oestrogens that could be expected to help prevent or to successfully treat breast cancer. It has been reported that the risk of breast cancer in western societies is indirectly proportional to the level of phyto-oestrogens in the diet and to the amounts of phyto-oestrogen metabolites excreted in the urine.

Cancer of the prostate generally is considered to be associated with sex hormone dysfunction and the growth of prostatic cancer cells is influenced by oestrogens and androgens. The incidence of prostatic cancer is low in communities with high legume intake and, conversely, is high in Western socieites. Phyto-oestrogens are though to protect from development of prostatic cancer. One mechanism may be the effect of phyto-oestrogens on lowering the proportion of unbound:bound reproductive hormones in the blood. However, there is other evidence to suggest that phyto-oestrogens, particularly isoflavones, can have a direct influence on intracellular enzymes within prostatic cells.

Pre-menstrual syndrome has uncertain aetiology and pathogenesis, although most certainly is

associated with reproductive hormone dysfunction. It also is a syndrome which has reportedly lower incidence in communities maintaining traditional high-legume diets. It is proposed that phyto-oestrogens will alleviate this condition by restoring balance to oestrogen metabolism.

Menopausal syndrome is associated with changes in the oestrogen profile in the body with advancing age. Adverse clinical symptoms may be treated with oestrogen replacement therapy. There is evidence that foodstuffs high in phyto-oestrogens are a suitable alternative to synthetic hormones in this respect, producing alleviation of adverse clinical symptoms. Again, it is proposed that phyto-oestrogens will function by restoring balance to oestrogen metabolism.

~~Benign (fibrocystic) breast disease has unknown aetiology. However, its association in women~~ with certain stages of the menstrual cycle is strongly suggestive of oestrogen dysfunction. There currently is no successful treatment of this condition. Phyto-oestrogens are proposed to successfully treat this condition by restoring balance to oestrogen metabolism.

Atherosclerosis is associated with cholesterol metabolism which in turn is associated closely with oestrogen metabolism. The generally higher incidence of atherosclerosis in young men versus young women, the rising incidence in women following menopause, and the lower incidence in post-menopausal women receiving oestrogen replacement therapy, all point to the modifying influence of oestrogens on cholesterol metabolism. A prime effect of oestrogens on cholesterol metabolism is stimulation of the liver to process cholesterol, particularly the highly atherogenic low-density lipoproteins and very low-density lipoproteins, into bile salts. It is proposed that phyto-oestrogens have an important hypocholesterolaemic effect in humans. There may be a variety of mechanisms involved, but one may be the stimulation by phyto-oestrogens of ~~cholesterol catabolism by the liver~~.

MODE FOR CARRYING OUT THE INVENTION

The invention is now described with reference to various examples.

EXAMPLE 1 - Preparation of Red Clover Product

Tablets were prepared using red clover in accordance with the following procedure. The raw plant material is harvested and dried; such drying being either sun-drying or from applied heat.

separa for use in human and animal foodstuffs. The hypocotyls normally are not separated and p.. rocessed along with the cotyledons. However, a small number of soybean processors are se ating hypocotyls by the above methods in order to reduce the astringent taste of soyfle.. for human consumption, and currently these hypocotyls are either discarded or proces to flour for use in animal feed.

EX.. E 3 - effect of administering red clover extract to humans (not of the invention)

Seve.. mal individuals were studied for the comparative effects of red clover extract and whole .. mes on blood cholesterol levels. All the individuals were consuming a standard Weste.. iet with minimal levels of legumes.

Thre.. consumed between 100-150 g haricot or navy beans daily for 3 weeks as a supp.. to their normal diet. This yielded an approximate daily isoflavone dosage or betwe.. -100 mg.

Four .. individuals (3 men, 1 woman) consumed 5 g of red clover extract containing 100 mg is.. nes daily for 3 weeks.

Total .. cholesterol levels were determined immediately before and immediately following the c.. ge.

	Pre-treatment	Post-treatment	% change
Beans			
Patien..	5.77	5.46	- 5.4
Patien..	6.24	6.12	- 1.9
Patien..	7.45	8.51	+14.3
Red .. extract			
Patien..	6.53	5.90	- 9.6
Patien..	7.43	6.63	-10.8
Patien..	6.33	5.50	-13.1
Patien..	6.98	7.28	+ 4.3

The .. ver extract had a significantly ($P < 0.05$) greater hypocholesterolaemic effect than